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Enteroviruses of Swine. III. Studies on Experimental Infection in Pigs

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WE REPORTED previously on the detection and identification of an enterovirus of swine [2] and on some aspects of the natural history of infection in a herd of swine.[5] These studies indicated that a solitary serotype of swine enterovirus was widespread in these pigs and in their habitat. We were unable to associate any illness in swine with the presence of enterovirus in the alimentary tract. The infection was apparently restricted to susceptible cells of the lower alimentary tract, and this infection was followed by the development of specific antibodies. However, active immunity apparently developed before passively acquired antibodies were lost, and in the earlier studies we were unable to reveal the point in the lives of young pigs when active immunization took place. Furthermore, there was a latent period during which excretion of enterovirus was delayed in suckling pigs. The evidence strongly suggested that antibodies in colostrum prevented alimentary infection for a brief period in very young pigs, but whether these antibodies prevented generalized spread of virus in other body tissues could not be determined in these earlier studies.

In order to answer these and other questions, 36 pigs that had not received colostrum were infected experimentally with swine enterovirus, while 9 similar animals were used as uninfected controls.* These studies were begun in order to learn whether the Municipal Farm (MF) strain of enterovirus has an apparent or inapparent pathogenic effect on young pigs, particularly with reference to (*a*) bringing about illness and death, (*b*) the invasiveness of the virus for certain tissues both in and outside the alimentary tract, and (*c*) temporal relationships between the experimental infection and acquisition of specific neutralizing antibodies.

MATERIALS AND METHODS

Four sets of experiments involved three different environments: the Municipal Farm, where pigs were exposed to infection with enterovirus present in the habitat; open laboratory pens, located in an area where no previous work had been done with swine enterovirus; and isolation units [6] located in the Department of Animal Pathology at the University of Nebraska.

Two major experiments form the basis of the report; they are reported in detail. The two minor experiments are briefly summarized at the end of this report.

Virus.—The same stock of the prototypic MF strain of virus used in earlier studies [5] was employed in these experiments. This "purified" virus

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* Included in the control group was 1 pig which suckled the sow (*see* Results section).

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stock had an infectivity titer of $10^{7.5}$ t.c.d.$_{50}$ per milliliter in cultures of fetal pig kidney cells.

Municipal Farm.—Immediately after birth, 8 pigs in one litter were placed in a pen adjacent to the sow. They were fed a diet consisting of modified cows' milk [7] containing oxytetracycline (8.75 mg. soluble powder per quart). A single pig from this litter remained with the dam and received colostrum.

Open Pens.—At birth, 15 pigs were delivered into sterile canvas bags. These pigs were taken immediately to laboratory pens located in a hospital. Two rooms were used; infected pigs were quartered in four pens in one room and control pigs were placed in a single pen in the other room.* The same diet used at the Municipal Farm was fed these pigs. The 15 pigs were allotted into five groups each containing 3 pigs. On the day after birth, pigs in four groups were inoculated with varying doses of MF strain virus by nasal-oral, subcutaneous (s.c.), intraperitoneal (i.p.), and intracerebral (i.c.) routes, respectively (table 1). The 3 pigs of the same age, housed in the room with the single pen, were inoculated i.p. with 5.0 ml. of sterile maintenance medium.[2] Pigs were observed twice daily for evidences of illness. Each pig's body temperature (rectal) was obtained daily during the life of the animal. Fecal samples were obtained daily, and blood samples and nasal secretions were obtained on the third and fifth days for viral and antibody studies.

Isolation Units.—The 21 pigs obtained by hysterectomy [8] were immediately placed in individual isolation units. These units were designed to maintain colostrum-deprived pigs in a disease-free state, or, in the case of experimentally infected animals, to prevent cross-infection by airborne, oral, or contact routes. The diet of these pigs consisted of modified cows' milk used in other experiments, but without any added antibiotic.

The first two litters consisted of 16 pigs. When the pigs in these two litters** were six and nine days old, 12 were given $10^{5.5}$ t.c.d.$_{50}$ of enterovirus by the nasal-oral route while anesthetized lightly with ether. Four pigs, 2 from each litter, were similarly given sterile nutrient broth (table 1). On the second, fourth, sixth, and eighth days after nasal-oral inoculation, 2 infected pigs were killed; on the ninth and tenth days, 1 pig was killed daily. The last 2 pigs were killed 29 days after they were given virus. The 4 control pigs were killed on the eighth (2 pigs), ninth (1 pig), and tenth (1 pig) days. Feces and blood were obtained daily during the life of each pig and again at necropsy. Body temperatures were measured daily also, and blood (total and differential leukocyte counts) studies were made every second or third day.

Since 1-day-old pigs had been used in the previous study done in laboratory pens, another group of 4 pigs, 1 day old, were given virus; 1 pig from the same litter was given nutrient broth. The dose, the route, and the method of administration were the same as described for other pigs kept in isolation units. These 5 pigs were examined daily (including temperatures); they were not killed during the ten-day period of observation.

Necropsy Studies.—Many different tissues were obtained for histological examination and for the detection of enteroviruses, using methods described previously.[5] Tissue samples obtained for histologic studies were fixed in 10 per cent formalin and stained with hematoxylin and eosin. Tissues for virus studies were obtained by a sterile surgical technique; they were stored in a dry-ice box until they could be tested.

Virus and Antibody Studies.—Feces, nasal secretions, blood, serous fluids, and tissues collected at necropsy were tested for presence of virus, and for the amount of virus present in positive samples. The methods used for preparation of samples for tests; for preparation, growth, and maintenance of fetal pig kidney tissue cultures; for determining the concentrations of virus and antibody; and the criteria used for detecting enterovirus in tissue cultures have all been described previously.[2, 4, 5]

RESULTS

Clinical Evidence of Infection.—Pigs in Laboratory Pens.—The 15 pigs in this study were apparently healthy. The average rectal temperature on the first day of life was 102.1 F. Within 24 to 72 hours, the 12 pigs which were fed the MF strain became ill. Pigs inoculated i.p. and s.c. developed the earliest signs of illness. Within 24 hours, their body temperatures rose and they were listless and inactive. Pigs inoculated by other routes developed similar signs of illness when they were 48 to 72 hours old. Within 12 hours from onset of illness, many pigs lay on their sides, squealed when jostled, failed to eat, and, if they ate, quickly vomited. Respiration was rapid and grunting; coarse bubbling râles were heard on auscultation of the chest. These signs lasted for about 48 hours. Rectal temperatures ranged between 103.0 and 105.8 F.; the mean temperature for all infected pigs was 104.8 F., compared with 102.5 for control animals. During this early period of illness, 5 pigs died (table 1).

By the third day of illness, the 7 infected pigs that were still alive were dehydrated and weak. The haircoats appeared dull and rough. Respiratory distress continued; pigs did not eat. Diarrhea intervened; stools were watery and yellow or gray. Incoordi-

* The swine caretaker was instructed to change boots and clothing when passing between rooms; however, these precautions proved to be inadequate to prevent transport of the virus from inoculated to control pigs.

** The pigs in these litters were delivered three days apart; each litter occupied units in a different room. When 2 pigs were killed on the same day, 1 animal from each litter was killed.

TABLE 1—Summary of Data on Illness in Pigs Inoculated with the MF Strain of Swine Enterovirus

No. pigs	Age when inoculated (days)	Inoculum			Day of onset of illness	No. of pigs with signs of illness								Day of death
		Route	Volume (ml.)	Log_{10} $(c.c.d_{50})$ per ml.		Fever*	Listless, weak, inactive	Labored breathing	Prostrated	Dehydrated	Vomiting	Diarrhea	Convulsions	
LABORATORY PENS														
3	1	oral	5.0	7.53	3, 3, 2	3	3	3	2	1	1	1	1	3, 4, S
3	1	s.c.	5.0	7.53	1, 1, 1	3	3	3	3	2	1	2	0	3, 4, 8
3	1	i.p.	5.0	7.53	1, 1, 1	2	3	3	2	1	1	0	1	3, 3, 8
3	1	i.c.	0.5	7.53	2, 1, 2	3	3	3	3	1	1	1	2	3, 4, 5
3**	1	i.p.	5.0	.....	.....	1	1	0	0	0	0	0	0	S, S, S
ISOLATION UNITS														
12	6 or 9	oral	1.0	5.5	.....	0	0	0	0	0	0	7 ±†	0	.....‡
4**	6 or 9	oral	1.0	....	.....	0	0	0	0	0	0	3 ±†	0	.....‡

S = survived. * Body temperature ≧ 2 F. above that observed at the time of inoculation. ** Controls. † Soft stools of mucous consistency; not true diarrhea. ‡ These pigs were killed—*see* text for scheduled days.

nate, jerking movements of the limbs occurred in 4 pigs. Fever continued; the mean body temperature was 103.9 F. Four of these pigs died four to five days after inoculation. Only 3 survived the first week of infection. By the sixth day, illness appeared less intense; the 3 surviving pigs became more active, diarrhea lessened, and appetite reappeared. One pig, however, continued to have respiratory distress and died on the eighth day. The other 2 pigs appeared normal on the eighth day, the final day of observation. The 3 control pigs remained healthy; enterovirus was not detected in fecal samples obtained during the experimental period.*

Pigs in Isolation Units.—The 16 pigs were apparently healthy prior to being given virus, when they had rectal temperatures of 103.3 to 105.7 F. (mean, 104.4 F.).

None of the 12 infected pigs in these two litters and none of the 4 pigs inoculated with nutrient broth had significant changes in body temperature, significant deviations in leukocyte counts, nor any clinical signs of illness during the experimental period. Soft feces excreted by 7 infected pigs were not considered to be related to infection by enterovirus, since similar excreta were observed in 3 control pigs. The clinical findings for 31 infected and control pigs are summarized (table 1).

Abnormalities Seen at Necropsy.—Pigs in Laboratory Pens.—Five pigs which died on the third day after infection were found to have 5 to 15 ml. of fluid in several of the serous body cavities. The effusions in the thoracic and peritoneal spaces were thin, straw-colored, and cloudy or serosanguinous. The fluid in the pericardial sac was clear and straw-colored. These fluids were not purulent. Fibrinous strands adhered to parietal and visceral pleura and between peritoneal reflections in 4 of these pigs. The stomach and intestines were bloated. Petechial hemorrhages were seen in mucous membranes of the fundic portion of the stomach of 1 pig. In 2 other pigs, portions of the mucous membrane were separated from the submucosa. In contrast, the mucous membranes of the intestines appeared normal. The contents of the colon were black and fluid. Petechial hemorrhages

* Two of these 3 control pigs excreted enterovirus on the ninth day after intraperitoneal inoculation with nutrient broth. By this time in the lives of these pigs they did not experience illness as a result of infection presumably acquired by cross contamination.

were observed in the mucosa of the urinary bladder. Except for the fibrinous exudates present on the visceral surfaces of organs, no abnormalities were seen in the lungs, hearts, livers, spleens, kidneys, adrenals, or mesenteric, axillary, inguinal, and cervical lymph nodes obtained from these 5 pigs.

The 5 pigs which died four to eight days after inoculation had similar lesions which generally were more advanced than those just described, except that effusions in serous cavities were less marked, probably due to the dehydrated condition of the animals. No free fluid was observed in the serous cavities of 3 pigs; less than 5 ml. of brown, cloudy fluid was present in 2 pigs. Fibrinous adhesions bound thoracic and abdominal organs to each other, and extended to the parietal membranes. In 2 pigs, ulcers were observed in the mucous membranes of the fundic portion of the stomach. The jejunal mucous membranes of 3 pigs were hyperemic, especially along the sides opposite the visceral mesenteric attachment. In these 5 pigs, the contents of the jejunum were fluid. In 2, the fluid contents were grey-green, and in 3 they were yellow-green. The colons' contents were also fluid and dark green or black. Mesenteric lymph nodes from 3 pigs were about twice normal size and had subcapsular hemorrhages. Focal areas of pulmonary consolidation were seen in 2 pigs on the fifth and eighth days of the study.

Bacteria, chiefly enteric (*Escherichia coli*, *Aerobacter aerogenes*, and Proteus spp.), were cultured from thoracic and peritoneal fluids, intestinal contents, and suspensions of tissues prepared from brain, liver, kidneys, and lymph nodes. Streptococci and staphylococci (species undetermined) were cultured also from several tissue extracts. Pleuropneumonia-like organisms (PPLO) and fungi were not cultivated in veal heart infusion broth or in Sabouraud's agar inoculated with body fluids, exudates, or tissue extracts.

Pigs in Isolation Units.—Abnormalities specifically related to infection from enterovirus were not observed. Edema of the interstitial tissues between the coils of the spiral colon was noticed in 8 infected pigs and 4 control pigs. Similar findings have been observed in colostrum-deprived pigs maintained in isolation units.* Black spots, similar in appearance to petechial hemorrhages and numbering 2 to 50 per pig, were present in the mucosal surfaces of the urinary bladders of 5 infected and 4 control pigs. About 15 ml. of blood-tinged fluid was present in the right half of the thorax of 1 pig killed on the sixth day; the right lung was atelectatic. Probably the intrathoracic changes observed in this pig were acquired before or shortly after birth. Otherwise, the alimentary tract and the heart, lungs, liver, spleen, adrenals, and lymph nodes were normal in appearance. None of the tissues, normal or abnormal, were cultured for bacteria, PPLO, or fungi. The kinds of lesions observed in experimentally infected pigs are summarized (table 2).

Histologic Studies of Tissues.—Pigs in Laboratory Pens.—Many different tissues

* Young and Underdahl[9] consider this abnormality to be associated with the low serum protein level of these pigs caused by the lack of gamma globulin in the serum.

TABLE 2—Frequency of Abnormalities in Pigs Experimentally Inoculated with the MF Strain of Swine Enterovirus

Abnormalities	Laboratory pens					Isolation units	
	Oral	s.c.	i.p.	i.c.	Cumulative score	Virus	Broth
Increased thoracic fluid	1/2	0/3	2/2	1/3	4/10	1/12	0/4
Fibrinous thoracic adhesions	0/2	3/3	2/2	2/3	7/10	0/12	0/4
Pulmonary consolidation	0/2	1/3	0/2	1/3	2/10	0/12	0/4
Increased pericardial fluid	1/2	0/3	2/2	0/3	3/10	0/12	0/4
Gaseous distension of stomach	1/2	0/3	2/2	3/3	6/10	0/12	0/4
Gastric mucosal hemorrhage	1/2	2/3	2/2	1/3	6/10	0/12	0/4
Increased peritoneal fluid	1/2	0/3	0/2	1/3	2/10	0/12	0/4
Fibrinous peritoneal adhesions	1/2	3/3	2/2	2/3	8/10	0/12	0/4
Intestinal mucosal hemorrhage	1/2	1/3	0/2	1/3	3/10	0/12	0/4
Interstitial edema in spiral colon	0/2	0/3	0/2	0/3	0/10	8/12	4/4
Petechiae of urinary bladder	0/2	0/3	1/2	0/3	1/10	5/12	4/4
Mesenteric lymphadenitis	1/2	1/3	0/2	1/3	3/10	0/12	0/4

Numerator = No. of pigs with lesions; denominator = No. of pigs in the group.

The 3 control pigs inoculated i.p. with maintenance medium were examined at necropsy; however, they were not killed until the 62nd day after inoculation. No abnormalities were seen in organs of these animals.

were involved in the inflammatory process. Focal areas of necrosis were present in the mucosa of the intestine; the submucosa and muscularis mucosae adjacent to these areas were infiltrated with polymorphonuclear cells and lymphocytes. Bacteria were seen in suppurative areas and within the lumina of blood vessels. Acute suppurative pleuritis and peritonitis were common findings. In the lungs, the alveolar walls were hyperemic and edematous, proliferation of septal cells was observed, and, in a few of the pigs, blood and fibrin were present in alveoli. Acute splenitis was present. The mesenteric lymph nodes had lesions of hemorrhagic lymphadenitis. Hyperemic embryonal glomeruli, probably accounting for previously recorded "petechiae," were seen in subcapsular areas of the kidney. Acute suppurative meningitis involving brain, brain stem, and spinal cord was most pronounced in pigs inoculated intracerebrally, but meningitis was also encountered in pigs inoculated by other routes. This meningitis was not primarily viral in origin, since enterovirus was detected in the brain of only 1 pig (table 6) and might have been remnant from the inoculum. Abnormal histologic changes (except those already mentioned) were not seen in the hearts, livers, kidneys, or urinary bladders of these pigs.

Pigs in Isolation Units.—The kind of inflammatory response observed in these pigs was quite different from that observed in infected pigs kept in laboratory pens. The lesions in the alimentary tract were less severe, and were confined to the jejunum and colon. Catarrhal enteritis of the jejunum was characterized by hyperemia of villi, with large number of leukocytes in the submucosa and within the lumen. The colon was more severely involved by catarrhal and suppurative enteritis. The epithelia on the tops of villi were desquamated, goblet cells appeared to be more numerous than ordinarily seen, and large numbers of leukocytes and lymphocytes were present in both submucosa and lumen. Small numbers of bacteria were present in areas of suppurative enteritis and within the lumen of the colon.

The extent of the injury was related to the duration of the infection. Acute inflammatory changes were most intense in pigs killed earliest (2 to 4 days); in pigs killed later (8 to 10 days), the lesions were more proliferative; e.g., hyperplasia of lymph nodules of the jejunum. Acute interstitial hepatitis was found in pigs killed after eight to ten days. Serous adenitis was seen in mesenteric lymph nodes during the early phase of infection; later, hyperplasia of lymph follicles and subcapsular hemorrhage were seen. Otherwise, the histologic anatomy of the lungs, heart, spleen, urinary bladder, and brain was normal. Lesions similar to those described for infected pigs were also observed in the normal control pigs.

Fecal Excretion of Virus.—Pigs in Laboratory Pens.—Within 48 hours after they were given virus, enterovirus was present in feces from 3 pigs (inoculated i.p., i.c., or orally). By 72 hours, virus was found in feces from 4 of the 5 pigs which died soon thereafter, and from 6 of the other 7

TABLE 3—Enterovirus in Feces Collected Daily from Experimentally Infected Pigs

Age of pigs (days)	Route of inoculation	Inoculum $\log_{10}$ t.c.d.$_{50}$ per pig	Postinoculation day									
			1	2	3	4	5	6	7	8	9	10
ISOLATION UNITS												
6 or 9	oral	5.5	3/12	7/12 (3.2)*	10/10	10/10 (4.2)*	7/8	8/8 (5.0)*	6/6	5/6 (3.2)*	4/4 (4.5)*	3/3 (0)**
6 or 9	oral	....	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/2	0/1
LABORATORY PENS												
1	oral	8.2	0/3	1/3	1/3	0/2	1/2	1/1	1/1	1/1	.....	.....
1	i.c.	7.2	0/3	1/3	2/3	2/2	1/1	.....	.....	.....	.....	.....
1	s.c.	8.2	0/3	0/3	1/3	2/2	2/2	1/1	1/1	1/1	.....	.....
1	i.p.	8.2	0/3	1/3	2/3	1/1	1/1	1/1	1/1	1/1	.....	.....
1	oral	....*	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	.....	.....
No. of inoculated pigs excreting virus			3/24	10/24	16/22	15/17	12/14	11/11	9/9	8/9	4/4	3/3
Totals for both groups (%)			12	42	73	88	86	100	100	89	100	100

Numerator = No. of pigs excreting virus; denominator = No. of pigs tested.

* Geometric mean titers of virus recovered from feces collected at necropsy of pigs killed on indicated days. ** The (0) indicates that virus was found on the rectal swab but not in postmortem feces of the single pig killed on that day.

Daily fecal samples collected from 2 infected pigs kept for 29 days yielded 24 and 25 positive tests. Postmortem fecal samples had a mean titer of $10^{5.5}$ t.c.d.$_{50}$/0.1 Gm.

surviving pigs as well. The time and rate of appearance of virus in feces was apparently not a function of the route of inoculation (table 3), since virus appeared at about the same time in feces of pigs infected by any of the four routes employed in this study.

During the eight days of observation, enterovirus was not found in feces of the 3 control pigs. However, control pigs began to excrete virus on the day following the close of this study. No clinical manifestations of illness accompanied the infection in these pigs. The control pigs were used for other purposes (e.g., to obtain hyperimmune serum against the MF strain) and were ultimately killed at 2 months of age. Abnormalities were not found at necropsy.

Pigs in Isolation Units.—Of the 12 infected pigs, 3 began to excrete virus within 24 hours, 5 within 48 hours, and 3 within 72 hours. Only 1 pig, killed after 96 hours, did not yield virus in the daily fecal samples. However, a larger sample of feces removed from this animal at necropsy contained enterovirus. After its first appearance, virus was detected almost without exception in the successive daily samples of feces (60 to 67 samples were positive). Feces were obtained daily from 2 pigs which survived 29 days; following onset of virus excretion in these pigs, enterovirus was detected in 49 (89%) of 55 rectal swabs. The 4 control pigs did not excrete enterovirus in any of the fecal samples obtained daily for ten days.

The concentration of virus in feces was determined only for samples obtained at necropsy from the terminal colons of pigs kept in isolation units. The mean concentrations of virus in feces increased from $10^{3.2}$ t.c.d.$_{50}$/0.1 Gm. of feces from the 2 pigs killed on the second day to $10^{5.0}$ t.c.d.$_{50}$/0.1 Gm. from the 2 pigs killed on the sixth day. Since each of these pigs was given $10^{5.5}$ t.c.d.$_{50}$ of MF strain, viral multiplication clearly occurred in the alimentary tracts of infected pigs, either wholly within these tissues or in other organs from which virus was recovered. A summary of the recovery of enterovirus from fecal samples collected daily from experimentally infected newborn pigs is given (table 3).

Absence of Virus in the Nasopharynx.—Enterovirus was not detected in the nasopharynx of the 12 infected (24 samples) or of the 4 control (8 samples) pigs infected in the laboratory pens.

Distribution of Virus in Tissues of the Alimentary Tract.—Pigs in Laboratory Pens.—Segments of the alimentary tract were obtained from 10 of 12 infected pigs. Virus was found in the stomachs and small intestines of a few of the pigs inoculated by several routes, but tests pertaining to pigs inoculated intraperitoneally (table 4) were negative. The concentrations of virus increased slightly in more distal segments of the intestinal tract; the largest concentrations were obtained from the spiral and terminal segments of the colon.

Pigs in Isolation Units.—The results differed from those for the preceding pigs in that virus was not found in segments of stomach or duodenum obtained from 12 infected pigs. However, large quantities of virus, $10^{1.5}$ to $10^{6.0}$ t.c.d.$_{50}$/0.1 Gm., were recovered from the washed wall of the colon or from the contents of the colon (table 5).

The Distribution of Virus in Tissues Outside the Alimentary Tract.—In Laboratory Pens.—The various tissues obtained

TABLE 4—The Kinds of Tissues from Which Enterovirus Was Recovered in the Alimentary Tracts of Pigs Inoculated by Several Routes and Kept in Laboratory Pens

Tissues or contents	Route of inoculation				Cumulative scores	
	Oral	s.c.	i.p.	i.c.	No. positive	Mean titer*
Stomach	N, 3.0	3.5, N, N	N, N	N, 3.0, N	3/10	3.2
Duodenum	ND, 2.0	N, ND, ND	ND, ND	ND, 2.5, ND	2/3	2.2
Jejunum	N, 4.0	2.5, N, N	N, N	N, 2.0, N	3/10	2.8
Ileum	ND, 4.5	2.5, ND, ND	ND, ND	ND, 3.5, ND	3/3	3.5
Spiral colon	+, 2.5	4.0, +, +	+, +	N, 4.5, +	9/10	3.7**
Terminal colon	N, 4.0	3.0, +, +	N, +	N, 5.5, +	7/10	4.2**
Day of death	3, 4	3, 4, 8	3, 3	3, 4, 5	.....	....

N = negative at $10^{-1.0}$ dilution. ND = not done. + = virus isolated but not titrated.
* Expressed as $\log_{10}$ t.c.d.$_{50}$ per 0.1 Gm. of tissue or 0.1 ml. fluid.
** Geometric mean titer based on three samples.

TABLE 5—Tissues and Contents of the Alimentary Tract from Which Enterovirus Was Recovered in Pigs Inoculated by the Oral Route and Kept in Isolation Units

Tissues or contents	Day killed					
	2	4	6	8	9 to 10	29**
Stomach	N,* N	N, N	N, N	N, N	N, N	N, N
Duodenum	N, N	N, N	N, N	N, N	N, N	N, N
Duodenal contents	N, N	N, N	N, N	N, N	N, N	N, N
Jejunum	N, N	2.5* N	N, N	N, N	N, N	N, N
Jejunal contents	N, 3.0	N, N	N, N	N, N	N, N	N, N
Spiral colon	2.5, 1.5	4.5, 4.0	4.5, 1.5	4.5, 4.5	3.5, 4.5	6.0, 4.0
Spiral colon contents	2.5, 5.5	6.0, 6.0	4.0, 2.5	6.0, 5.5	6.0, 5.5	5.5, 6.0
Terminal colon	2.5, 3.0	2.5, 4.0	3.5, 3.0	N, 3.5	3.0, N	4.5, 3.0
Terminal colon contents	3.5, 3.0	3.5, 5.0	5.5, 4.5	2.5, 4.0	4.5, N	6.5, 4.5

N = negative at the $10^{-1.0}$ dilution. * Expressed as the $\log_{10}$ t.c.d.$_{50}$ per 0.1 Gm. of tissue or 0.1 ml. of fluid. ** The ileac portion of the intestine was also tested in the 2 pigs surviving 29 days. The washed tissues were negative at the $10^{-1.0}$ dilution, as were the ileac contents in 1 pig. Ileac contents in the other pig had a titer of 4.5.

from the 10 infected pigs that became ill and died, and those which yielded enterovirus, are listed (table 6). Viremia was detected in 2 pigs on the third and fifth days after inoculation. Other tissues from which enterovirus was infrequently recovered included mesenteric lymph nodes, liver, spleen, kidneys, urinary bladder, brain, and serous exudates. The concentrations of virus in these various tissues ranged from $10^{1.5}$ to $10^{3.5}$ t.c.d.$_{50}$/0.1 Gm. or 0.1 ml. For several tissues (marked +), virus concentrations were not determined.

Pigs in Isolation Units.—In general, similar results were obtained. Enterovirus was detected infrequently in mesenteric lymph nodes, livers, spleens, urinary bladders, and lungs from 5 of these 12 pigs. In contrast with pigs infected in laboratory pens, virus was not recovered from the blood (sampled daily during life and again at necropsy) or kidneys of these pigs. However, virus was detected in the lungs of pigs in isolation units; none was found in the lungs of the pigs in laboratory pens. The presence of virus in the lungs may represent virus aspirated during inoculation by the nasal-oral route. The results of these studies are given (table 7).

The Primary Immune Response.—None of the 31 pigs (infected and controls) in laboratory pens or in isolation units had demonstrable antibodies neutralizing the MF strain virus at the time of inoculation. In the laboratory pens, none of the 10 infected pigs that died after 3 to 8 days developed specific antibodies, nor did the 2 pigs surviving infection possess similar antibodies by the eighth day after inoculation. Six of 12 infected pigs kept in isolation units developed specific neutralizing antibodies. Four pigs killed after eight to ten days had serum dilution end points of 1:4 to 1:64. The 2 pigs killed on the 29th day had serum dilution end points of 1:64 and 1:160 (table 7). The 7 control pigs

TABLE 6—The Kinds of Tissues from Which Enterovirus Was Recovered Outside the Alimentary Tracts of Pigs Inoculated by Several Routes and Kept in Laboratory Pens

Tissues or body fluids	Route of inoculation				Cumulative scores	
	Oral	s.c.	i.p.	i.c.	No. positive	Mean titer*
Blood	N, N	N, N, N	+, N	N, N, +	2/10	ND
Lung	N, N	N, N, N	N, N	N, N, N	0/10	....**
Heart	N, N	N, N, N	N, N	N, N, N	0/10	....**
Liver	N, N	N, N, N	N, N	N, 1.5, N	1/10	1.5
Spleen	N, 1.5	N, N, N	N, N	N, N, N	1/10	1.5
Kidney	N, 2.0	3.5, N, N	N, N	N, 2.5, N	3/10	2.7
Urinary bladder	ND, 1.5	N, N, N	N, N	ND, ND, N	1/7	1.5
Mesenteric lymph nodes	N, 3.0	2.0, N, N	N, N	N, N, N	2/10	2.5
Brain	N, N	N, N, N	N, N	N, +, N	1/10	ND
Pleural exudate	N, —	—, N, —	+, +	N, —, N	2/6	ND
Pericardial exudate	N, —	—, —, —	+, N	—, —, +	2/4	ND
Peritoneal exudate	+, —	N, N, —	N, N	N, 1.5, N	2/8	1.5†
Day of death	3 4	3, 4, 8	3, 3	3, 4, 5	.....	....

N = negative at the $10^{-1.0}$ dilution. ND = not done. Plus signs indicates that virus was isolated but not titrated. Minus sign indicates that no exudate was present.

* Expressed as the $\log_{10}$ t.c.d.$_{50}$ per 1.0 Gm. of tissue or 0.1 ml. of fluid. ** Not pertinent. † Based on titration of one of two isolates.

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entered in these two studies did not have specific antibodies (14 dilution) by the eighth or tenth days when the studies were ended.

Résumé of Two Other Studies.—One-Day-Old Pigs in Isolation Units.—Studies were carried out with 1-day-old pigs in order to determine whether differences observed in the two preceding experiments were influenced by age of the pigs at the time of infection. Of 5 pigs, 4 were given the MF strain ($10^{5.5}$ t.c.d.$_{50}$) by the nasal-oral route and 1 was fed nutrient broth. Except for intervention of diarrhea in infected and control pigs, no other evidence of clinical illness was seen. These pigs had the same kind of inapparent infection reported for the slightly older pigs studied in isolation units.

Pigs at the Municipal Farm.—The 8 pigs kept in a pen adjacent to the dam became ill on the fourth day of life. All developed diarrhea, refused to eat, and became weak. The signs of illness were similar to those observed among infected pigs kept in laboratory pens. Four pigs died within 48 hours after onset of illness. By the sixth day, the remaining 4 pigs were recovering and by the tenth day they appeared healthy. The control pig which remained with the dam during this ten-day period had no signs of illness.

The 4 pigs that died had effusions in serous cavities, "bloating" of intestines, and fibrinous adhesions adhering to serous membranes of the thorax and abdomen. These abnormalities were similar to those seen in infected pigs kept in laboratory pens. In addition, gelatinous edema of peritoneal and mesenteric tissues was seen in 2 pigs. Enterovirus was recovered from pooled washed segments of the jejunum and colon of each pig that died. Enteric and other bacteria, including streptococci and staphylococci, were also present in tissues obtained from these pigs.

Enterovirus was first detected in feces from these pigs on the fourth (5 pigs), fifth (2 pigs), or sixth (1 pig) day of life. The 4 surviving pigs were still excreting virus on the tenth day of life.

TABLE 7—Tissues Outside the Alimentary Tract from Which Enterovirus Was Collected in Pigs Inoculated Orally and Kept in Isolation Units

Tissues	Day killed					
	2	4	6	8	9–10	29
Blood	N, N	N, N	N, N	N, N	N, N	N, N
Lung	N, 2.5	N, N	2.5, N	N, N	N, N	N, N
Heart	N, N	N, N	N, N	N, N	N, N	N, N
Liver	N, N	N, 2.5	N, N	N, N	N, N	N, N
Spleen	N, N	2.5, 3.0	N, N	N, N	N, N	3.5, N
Kidney	N, N	N, N	N, N	N, N	N, N	N, N
Urinary bladder	N, N	N, 3.0	3.0, N	N, N	N, N	3.5, N
Mesenteric lymph nodes	N, N	4.5, N	N, N	N, N	N, N	N, N
Brain	N, N	N, N	N, N	N, N	N, N	N, N
Serum antibodies	<4, <4	<4, <4	<4, <4	4, 16	64, 4	64, 160

N = negative at the $10^{-1.0}$ dilution. Serum titers are expressed as serum dilution end points using 100 t.c.d.$_{50}$ of the MF strain of virus.

DISCUSSION

After experimental inoculation with the MF strain of swine enterovirus, pigs deprived of colostrum developed active infection. Pigs kept in laboratory pens became ill and died; however, subsequent findings at necropsy indicated that these pigs also had an acute concomitant bacterial disease. In contrast, pigs kept in isolation units did not become ill; all except 1 of these pigs excreted large quantities of enterovirus in feces. Very probably, the risk of death for newborn pigs kept in laboratory pens was influenced markedly by the presence in many tissues of gram-negative bacteria. Other workers have shown that newborn pigs deprived of colostrum are more susceptible to serious illness from such infectious agents.[1,3] Our data suggested that swine enterovirus by itself was not sufficiently invasive to cause illness in newborn pigs. Thus, an inapparent infection was acquired not only in the native habitat of pigs,[2,5] but also after experimental transmission in the laboratory.

The lesions encountered in the tissues of pigs kept in laboratory pens probably were not specifically caused by the MF strain of

enterovirus, but were associated with the bacterial infection. Similar lesions were not observed in pigs kept in isolation units. Certain anatomical changes, such as petechial hemorrhages in bladder, and ulcers in the fundic portion of the stomach, are often encountered in newborn pigs dying of other causes. The absence of specific lesions in any tissue which could be associated with injury inflicted by swine enterovirus provided further evidence that this is a relatively avirulent agent for young pigs.

Nevertheless, this swine enterovirus is infective for very young pigs, multiplying primarily in tissues of the alimentary tract. The nasopharynx is probably not an important site of virus growth. No samples were taken from the oropharynx. Virus was irregularly detected in the walls of the stomach, duodenum, and jejunum. In sharp contrast, the colon always contained large quantities of virus, both in its wall and in its contents. This segment of the alimentary tract may be a preferential site of virus multiplication. However, it should be noticed that, for the few pigs tested, virus was also found in similarly high concentrations in the ileum. The data are inconclusive, however, as to which of these anatomical sites contains cells primarily or preferentially attacked by the virus. The large quantities of virus (as high as 10^7 t.c.d.$_{50}$/Gm.) detected in the wall of the colon cannot be accounted for by accumulation of virus (in crypts from which it could not be washed away) following its multiplication in the upper alimentary tract. The catarrhal and suppurative colitis observed in infected pigs therefore may be related to injury caused by virus or by a combination of virus and some other noxious agent (e.g., bacteria and toxins).

The concentrations of virus (per gram of tissue) obtained from the walls of the intestine during the first four days, and from the feces for as long as 29 days (the longest period tested), exceeded many times the amount of virus given to these pigs orally. Enterovirus also reached the alimentary tract and was excreted in feces after parenteral inoculation. Since it is unlikely that these pigs obtained virus by the oral route, it must have been transported, presumably in blood to the alimentary tract. Thus, the evidence supports the thesis made for primary multiplication in tissues of the lower alimentary tract. The occasional presence of virus in the stomach and duodenum is not satisfactorily explained, but the MF strain of virus may grow elsewhere in the alimentary tract, although the conditions necessary for such multiplication are unknown.

The MF strain was not confined strictly to the alimentary tract during the early phase of infection. Enterovirus was detected in lymph nodes, in blood, and in several parenchymatous organs. Virus may have reached lymph nodes by way of the lacteals, and may have reached the blood after traversing lymphatic sinuses. The presence of virus in the blood probably explained its presence in some other organs (e.g., liver and spleen). However, virus in the lungs may have been aspirated from regurgitated alimentary fluids, and virus in the brain was most likely due to remnants of the inoculum given intracerebrally. None of the findings indicated that the MF strain of virus produced primary encephalitis in nonimmune pigs.

The presence of virus in tissues adventitious to the alimentary tract was encountered only in pigs devoid of humoral antibody. Virus was never detected in these same tissues in naturally infected pigs[5] possessing antibody, or in experimentally infected pigs after such antibodies were acquired. However, these antibodies did not prevent infection of the alimentary tract, but antibodies obtained in colostrum by suckling delayed excretion of virus,[5] probably by preventing local multiplication of virus in the alimentary tracts of pigs for a brief period after birth.

Young pigs infected with the MF strain of virus acquired specific antibodies. Neutralizing antibodies appeared as early as eight days after nasal-oral inoculation in 6- to 9-day-old seronegative pigs. Similar antibodies did not appear within the same period in pigs infected at one day of age. Nonimmune pigs infected at the end of the first week of life appear to have an advantage over younger pigs in respect to speed of acquisition of antibodies. This finding confirms observations made in the preceding study.[5]

One incompletely assessed variable in these studies relates to the challenge dose of virus. Newborn pigs kept in laboratory pens were given as much as $5 \times 10^{7.5}$[3]

t.c.d.$_{50}$; whereas, newborn and slightly older pigs kept in isolation units were given 1 x $10^{5.5}$ t.c.d.$_{50}$ of the MF strain virus. The smaller dose initiated infection in pigs 6 to 9 days old and none of the pigs became ill; large quantities of virus were encountered in tissues of the alimentary tract.

TABLE 8—Comparison of the Distribution of Enterovirus in Tissues Obtained from Pigs with Experimental and Naturally Occurring Infections

Tissues or contents	Experimental infection		Natural infection	
	No. positive*	Mean titer**	No. positive	Mean titer
Blood	2/22	ND†	0/6	....‡
Lung	2/22	2.5	0/6	....
Heart	0/22	....	0/6	....
Liver	2/22	2.0	0/6	....
Spleen	4/22	2.6	0/6	....
Kidney	3/22	2.7	0/6	....
Urinary bladder	4/22	2.8	0/6	....
Mesenteric lymph nodes	3/22	3.2	0/6	....
Brain	1/22	ND	0/6	....
Stomach	3/22	3.2	0/6	....
Duodenum	2/15	2.2	0/4	....
Duodenal contents	0/12	....	0/4	....
Jejunum	4/22	2.8	2/6	2.2
Jejunal contents	1/12	3.0	1/6	1.5
Ileum	3/5	3.5	1/4	1.5
Iliac contnts	1/2	4.5	0/4	....
Spiral colon	21/22	3.8	4/6	3.9
Spiral colon contents	12/12	5.1	4/6	4.1
Terminal colon	17/22	3.5	6/6	2.8
Terminal colon contents	11/12	4.3	6/6	3.2
Serum antibodies	6/22	1:79	6/6	1:159

* Numerator = No. yielding virus; denominator = No. tested. ** Virus titers expressed as geometric mean of $\log_{10}$ t.c.d.$_{50}$ per 0.1 Gm. of tissue or contents. Serum titers expressed as 50 per cent serum dilution end point for 0.1 ml. serum vs. 100 t.c.d.$_{50}$ of MF strain. † ND = not done. ‡ Not pertinent.

Four newborn pigs, given this same dose of virus orally, remained healthy; probably they became infected, but it is uncertain that they did, since necropsies or virus and antibody studies were not done. Another uncertainty concerns whether newborn pigs kept in isolation units would develop clinical disease if given a dose similar to that given orally to pigs of the same age kept in laboratory pens. The 8 pigs deprived of colostrum developed infection on exposure to virus while in their native environment. Four of these pigs had patterns of infection similar to those observed for pigs of similar age kept in laboratory pens; 4 others had inapparent infections. Thus, dosage of virus or the antibiotic in the diet may determine the kind of infection acquired by young pigs, since one may potentiate and the other may diminish the susceptibility to fatal bacterial infection. At this point in these studies, these issues are controversial.

SUMMARY

Shortly after birth, 28 pigs deprived of colostrum were either fed or inoculated by parenteral routes with the Municipal Farm (MF) strain of swine enterovirus. Eight pigs removed from the dam at birth acquired natural infections. An additional 9 pigs were control animals.

Ten of 12 infected pigs kept in laboratory pens and 4 of 8 pigs acquiring infection in their native environment became ill and died. All developed active infection with enterovirus; coexisting generalized bacterial infections were found at necropsy. None of 12 infected pigs, 6 to 9 days old, nor 4 infected pigs, 1 day old, kept in isolation units developed recognizable illness. Twelve of these pigs did not have any abnormality at necropsy (or by histologic study of tissues) which was pathognomonic of infection by swine enterovirus. Although the 9 control animals remained healthy and abnormalities were not found at necropsy, 3 excreted enterovirus on the eighth day after induced infection of littermates.

Virus studies indicated that primary sites of viral multiplication were in tissues of the lower alimentary tract, particularly in the ileum and colon. Virus was recovered from the blood obtained from 2 pigs, and occasionally in other visceral organs. Humoral antibodies specifically neutralizing the MF strain of virus appeared about eight days after young pigs had been experimentally infected.

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SUMMARIO IN INTERLINGUA

Enterovirus Porcin. III. Studios in Infection Experimental de Porcos

Brevemente post nato, 28 porcos private de colostro recipeva—per via oral o parenteral—enterovirus porcin del racia MF (Ferma Municipal). Octo porcos, separate ab le matre a nato, acquireva le infection naturalmente. Novem porcos additional serviva como animales de controlo. Dece inter 12 inficite porcos in stabulos laboratorial e 4 inter 8 porcos inficite in lor ambiente natal se poneva malade e moriva. Omnes disveloppava active infectiones per enterovirus. Le co-existentia de generalisate infectiones bacterial esseva constatate al necropsia. Nulle del 12 inficite porcos con etates de inter 6 e 9 dies e nulle de 4 inficite porcos de etates de 1 die e mantenite in isolation disveloppava le morbo in forma recognoscibile. Dece-duo de iste porcos habeva nulle anormalitate al necropsia e nulle anormalitate histologic de genere pathognomonic pro infection per enterovirus porcin. Ben que le 9 animales de controlo remaneva san e revelava nulle anormalitate al necropsia, 3 de illos excerneva enterovirus le octave die post le inducite infection de fraternos del mesme portata.

Studios virologic indicava que sitos primari del multiplication de virus esseva presente in le tissus del vias alimentari inferior, particularmente in le ileum e le colon. Virus esseva retrovate in le sanguine de 2 porcos e a vices in altere organos visceral. Anticorpore humoral, neutralisante specificamente le racia MF del virus, appareva circa octo dies post que le juvene porcos habeva recipite lor infection experimental.

Influence of Heat Treatment on the Number of Ectopic Lymphoid Foci in Chickens

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LYMPHOID FOCI in the various organs of the chicken are regarded as normal by some investigators, particularly when the areas are small, few, and of common occurrence.[3, 6, 7, 10, 12, 23, 30] Palmer and Biely[25] carefully reviewed the significance of lymphoid accumulation in the nervous system of the chicken.

Our own studies have indicated that even small lymphoid areas invade and destroy adjacent tissue, and that sometimes they arise as plugs within vessels.[15–17, 19, 20] The small areas are histologically identical to larger areas involving as much as 30 per cent of the section.[15] Oakberg[22] found close agreement in the skewed shapes of the frequency curves for a number of lymphoid areas in the splenic nerve and for areas counted in the pancreas. Lucas[15] and Lucas and Oakberg[20] observed that there was no natural separation between so-called "normal lymphoid foci" and amounts that are generally regarded as microscopically diagnostic of lymphomatosis. Any separation into two categories is purely arbitrary. Too often, conclusions as to the normality of ectopic lymphoid areas represent subjective opinions based on observations of relatively few specimens from chickens of various ages, rather than on an adequate number of measurements that can be subjected to statistical analyses.

Payne and Breneman[27] reported a lymphoid bridge from the thymus gland, where the occurrence of lymphocytes is universally regarded as normal, to the thyroid gland where, to all appearances, the same cells entered and destroyed the tissues of this organ. Other endocrine glands also were severely invaded and damaged by lymphocytes. If this invasion by lymphocytes and the existence of numerous lymphoid areas is normal for chickens, then what criterion can be used for the abnormal growth if the lesion does not have the characteristics of a readily recognizable malignant neoplasm? Payne and Breneman[27] ask a similar question: "If the areas are not normal are they neoplastic in character?" They go on to say, "All are destructive of surrounding tissues, and glands may be completely replaced. When abnormal growths or lymphoid areas reach stages where they destroy entire glands, it seems to us, they might well be called neoplasms. If this conclusion can be accepted, then all lymphoid areas are potential neoplasms. As all gradations in size are present and as the cellular picture appears the same in all, can the line be drawn between small growths that are recognized as lymphoid areas and other larger growths which are clearly neoplastic?"

Jordan,[6] in his study on bone marrow, after concluding that lymphoid areas in this organ were normal hematopoietic structures, failed to give criteria other than arbitrary size and quantities of such areas that distinguished the normal from the leukemic condition.

A brief discussion of the terms "normal" and "abnormal" is necessary because some misunderstandings have occurred as to the scope of these terms. The reactivity of the host to infection or to stimuli from the environment is normal in all living organisms; pacifism on the part of tissues would lead to rapid annihilation. However, scars of the reactivity, allowing survival, cannot be considered normal. The scars and lesions are not part of ontogeny; they will not be transmitted to progeny. In the same sense, host reactivity to stimuli produces ectopic lymphoid foci. These are not seen in the embryo; they develop after hatching. King[11] defined normal concisely and accurately as " . . . that which functions in accordance with its design." The body is designed to respond to infection, but the results of those responses are lesions, and are not heritable or normal.

Pappenheim *et al.*,[26] Jungherr,[7] and Taliaferro and Taliaferro[30] recognized the occurrence of lymphoid areas as common in chickens and their interpretation agrees with our own; *i.e.*, that these areas represent host responses to various types of stimuli. We found that the etiologic agent or agents of the avian leukosis complex will increase the number of areas.[15, 19, 20, 23] Jungherr[8] observed a lymphoid response in cases of experimental sinusitis in turkeys.

In the hamster, sarcomas implanted under the skin caused numerous perivascular infiltrations throughout the body, in which hematopoieses could be found. Lymphoid nodules appeared in the liver. Kelsall and Crabb[9] regarded these as associated with the presence of the tumor under the skin and not metastases and not composed of tumor cells. The reactions seen in the hamster liver under these conditions resemble, as far as can be determined, the commonly observed conditions in the avian liver. In the mammal, this is obviously a host response and there is no doubt of the abnormality of the reaction, but the abnormality or normality of the same condition in the avian liver has been controversial for years. This is probably due to the fact that this type of reaction is common in chick-

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ens and because the causes of the reactions often cannot be identified. The mere fact that a histologic reaction is often repeated does not make it normal. King [11] said essentially the same thing: "The misuse of the term, normal, in a sense synonymous with "average" or "ordinary," is a case in point, for no such identity exists. The average may be, and very often is abnormal."

The objective of the present investigation was to find a physical therapeutic treatment that would produce a significantly lower lymphoid count in young chickens than in the controls. Reported here are the results from the use of heat.

MATERIALS AND METHODS

Five groups of inbred Single Comb White Leghorn chickens were involved in the test. Group 1, consisting of 30 chickens, provided a record of rectal temperatures from 1 to 29 days of age. Group 2 contained 50 chickens which served as unheated controls for groups 3 to 5.

The chickens in groups 3 to 5 (60 in each group) were given three, six, and nine heat treatments, respectively. Chickens of groups 2 to 5 were killed when they reached 35 days of age, and samples of liver and pancreas were taken for sectioning. Chickens of group 1 were discarded.

The data on rectal temperatures obtained from chickens in group 1 were assumed to be reliable measures of the normal body temperatures of chickens in groups 2 to 5 and, for that reason, are shown together (fig. 1). The chickens of group 1 were from the same inbred lines as those of groups 2 to 5. After hatching, they were brooded in the same room and in the same decks of the same battery as those of groups 2 to 5. Scheduling of the work load for the experiment did not allow for taking temperatures of the chickens given heat treatments.

Heat treatment consisted of placing chickens for 45 minutes in a 300-egg incubator. The average range of temperature for the incubator was 108.2 to 109.3 F. Lack of precision in thermostatic control occasionally allowed the temperature to vary from 108.0 to 110.0 F. Relative humidity, according to the instrument on the incubator, varied from 69.0 to 86.0 per cent with the average range of 73.1 to 78.2 per cent. Many chickens became prostrate from the treatment; some died, but most of them appeared unaffected. Those that were prostrate recovered after a few minutes to two hours.

Some damaging effects were observed at the temperature and humidity levels used. Moreng and Shaffner [21] conducted their survival studies at much higher temperatures and survival lasted 10 to 13 minutes.

The days on which the chickens were heat-treated in relation to their age are shown near the base line of figure 1.

The rectal temperatures were taken with oral clinical thermometers. Lamoereaux and Hutt [13] have shown the importance of inserting the bulb of the thermometer well into the body cavity.

After sectioning and staining liver and pancreas tissues, an ocular square enclosing 1.02 mm.^{2} of the field was moved at random over the slides 56 times, thus covering a total area of 57.12 mm.^{2} This sample size is twice the average cross-sectional area of the adult chicken pancreas. Lymphoid areas observed within the square or crossing the lower horizontal or left vertical boundary were counted; those crossing the remaining two lines were not counted.

RESULTS AND DISCUSSION

The normal body temperature of chickens is high compared to mammalian values. The average rectal temperature for 30 chickens is shown by the solid line in figure 1 and the 95 per cent fiducial limits by the dotted lines. The average rectal temperature one day after hatching was 104.2 F. This increased more or less steadily until about the fifteenth or sixteenth day. It was during the period of rising body temperature that heat treatments were given.

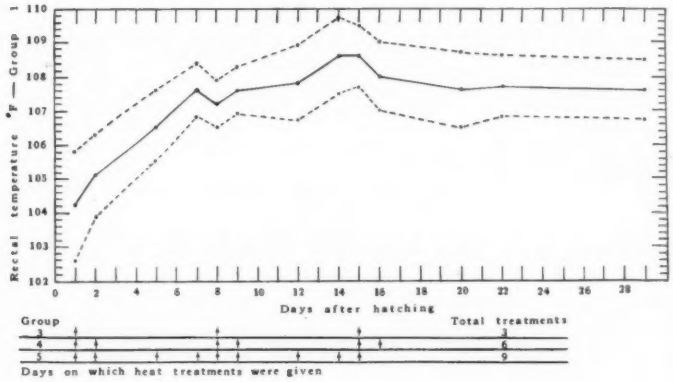

Fig. 1—Curve showing rectal temperatures for Single Comb White Leghorn chickens, from 1 to 29 days of age. Solid line is average temperature, the dotted lines are the 0.95 fiducial limits. Below the graph are arrows indicating the days on which chickens of the various groups were given heat treatments.

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The peak value of 108.6 F. at the fourteenth and fifteenth days was followed by a decline and leveling off to about 107.6 F., up to the 29th day when the records were terminated.

The rectal temperatures for group 1 chickens are considerably higher than those obtained by Bell.[1,2] He used a specially constructed thermocouple. His readings began on the eighth day after hatching and continued through the 31st. His data show a steady rise from the eighth to about the 23rd day, but at no time did his average points cross the lower limit of the 95 per cent fiducial interval in our data. Our chickens were brooded in a Maryland battery where the temperature under the heating element was 95 F. for the first two weeks. At this same brooding temperature, Scholes and Hutt[29] obtained a rise in body temperature (taken, as was ours, with a clinical thermometer) for the first eight days after hatching of 103.9 to 106.5 F. Thus, at the eighth day, Bell found 104.2 F., Scholes and Hutt, 105.8 F., Ram and Hutt,[28] 106.2 F.; and we recorded 107.2 F. No explanation can be given for the differences observed in records taken at three different laboratories. We used several thermometers and they were checked for agreement with each other. The experiment was conducted during February and March, before outdoor temperatures were high.

The summary of average number of areas tabulated is given (table 1), but, for the purposes of analyses, the values for each bird were subject to a log $(n+1)$ transformation, a method found to be suitable for this type of data.[18] The data (table 1) are given in arithmetic values, but these cannot be used directly for analyses because the frequency curves follow the same pattern discussed by Lucas and Oakberg,[20] and Oakberg.[22]

Heat treatment of young chickens reduced or retarded the development of ectopic lymphoid areas in the pancreas to an average of 64.3 per cent in the control chickens.

Variance analyses confirm what is indicated by the fiducial intervals (table 1). The control data for the pancreas were significantly higher (1% level) than for each treatment group. There was no significant difference between the three treatment groups; three treatments were as effective as nine.

The lymphoid host reactions of the liver paralleled those of the pancreas in that the highest values were obtained in the control group and all others were lower. Even the fluctuations among chickens in the treated groups corresponded for the two organs. However, the mean liver value for controls was not significantly higher than for any of the treated chickens, nor were means of the three treatment groups significantly different from each other.

Correlation analyses were made between pancreas and liver lymphoid foci to determine if reactivity was a function of individual organs within the bird or of the bird as a whole. The r value for the group means for liver and pancreas was +0.902 but, with 2 degrees of freedom, this was not significant at the 5 per cent level. Correlations within birds for these two organs gave the following results for each group: r equals +0.117 (group 2); r equals +0.208 (group 3); r equals −0.112 (group 4); and r equals +0.279 (group 5). None of these attained the 5 per cent level of significance.

Application of heat to recently hatched chickens definitely had an inhibitory effect on the development of the lymphoid foci in the pancreas. A similar reaction was noticed in the liver, although the difference between control and treated values was not large enough to be significant.

On a group basis, there was a high positive correlation between the reactions in the pancreas and liver. Within the individual

TABLE 1—Effect of Repeated Therapeutic Heat Treatments on the Number of Ectopic Lymphoid Areas

Group	No. of heat treatments	No. of chickens	Pancreas		Liver	
			Av. No. of lymph. areas*	0.95 fiducial interval	Av. No. of lymph. areas*	0.95 fiducial interval
2	Control	48	7.40	6.28–8.52	4.06	2.94–5.18
3	3	58	5.07	3.94–6.20	3.63	2.52–4.75
4	6	56	4.37	3.25–5.49	3.05	1.96–4.15
5	9	48	4.83	3.70–5.96	3.31	2.17–4.45

* The number of lymphoid areas found in a sample of 57.12 cmm. of sectioned tissue. Chickens were killed at 35 days of age.

bird the correlation was low, and in no case did it reach what is accepted as a significant level.

The temperature of the chicken normally is high after 2 weeks of age, but from 1 day to 2 weeks of age it was possible to apply heat several degrees above normal body temperature and still keep the bird alive.

The difference between normal body temperature and treatment temperature was actually so small that it was not anticipated that any significant results would be obtained, yet some reactivity was indicated, pointing to a sensitive mechanism of response in the young chicken.

If lymphoid area development is part of the chicken's normal ontogeny, then a response to heat treatment would not be expected; if it is part of the host's reaction to pathogens or even to what are generally considered to be nonpathogenic organisms, then any therapeutic treatment that would reduce the activity of these would be expected to elicit a reduced host response. The evidence, as far as it goes, supports the latter hypothesis and is in harmony with the evidence presented in some of the literature.

We do not yet have a satisfactory list of pathogens that will stimulate the body to produce ectopic lymphoid foci; in fact, we hardly have the beginning of such a list. It was mentioned earlier that the agent or agents of the avian leukosis complex was one such pathogen;[19] the organism that produces sacculitis and sinusitis in turkeys is another;[8] and it may be that bird malaria is a third, although it is difficult to compare our data with the results presented by Taliaferro and Taliaferro.[30] They did not take into account the increase in lymphoid areas with age, and did not take tissues from the controls at the same time they were taken from the treated birds, nor did they use any method of transformation on their data before testing for significance. They discarded as controls those chickens that had large amounts of lymphoid areas or those in which myelopoietic activity was involved.

From our limited data, aspergillosis in ducks appears to be a disease condition in which the amount of lymphoid tissue was not increased significantly above that found in ducks grossly negative for the disease.[16]

One question of considerable importance remains unanswered. Did the heat treatment reduce the number of lymphoid areas already produced, or did the heat reduce the amount of the agent that was stimulating the host? Information on this subject would require taking adequate samples from birds of several ages. We do not yet have a base line of average values for ectopic lymphoid accumulations associated with age.

Licht[14] has reviewed the history and effectiveness of heat therapy in mammals, particularly man. Olsen and Allen[24] reported that therapeutic heat treatments in turkeys were effective in assisting recovery from trichomoniasis. Scholes and Hutt[29] demonstrated that survival from *Salmonella pullorum* was greater in young, heat-treated chickens, and that resistance was greater in chickens that developed fever after infection than in those that did not. Ram and Hutt[28] found that high-temperature families were more resistant than low-temperature families to *S. pullorum*. However, Hutt[4] found that high summer heat (prolonged exposure compared to therapeutic use of heat) lowered the resistance of chickens to coccidia. Bell,[1,2] who used *Shigella gallinarum* as a test organism, found that during the several days following inoculation the resistant chickens had higher body temperatures than the susceptible ones; beginning several days later and continuing for the duration of the experimental period, the resistant chickens had lower body temperatures than the susceptible ones. Bell found that in noninoculated control chickens, the body temperatures of resistant birds were either the same or higher than those of susceptible birds, and concluded that high body temperature *per se* is not sufficient to account for genetic resistance to fowl typhoid.

Scholes and Hutt have discussed the effect of high temperatures on the interaction of host and invading organism, as has Bell,[2] who found that, under *in vitro* conditions, significantly greater phagocytic activity occurred in resistant than in susceptible lines of chickens. Ram and Hutt[28] associated high metabolism with high temperature and, in turn, with resistance.

SUMMARY

The rectal body temperature of the Single Comb White Leghorn chicken rises from an average of 104.2 F. one day after hatching to a level of 107.6 F. at 29 days of age. Values given at different laboratories for

the eighth day varied from 104.2 F. to 107.2 F.

When chickens, 1 to 16 days old, were subjected to three to nine heat treatments, each consisting of a 45-minute exposure to an external temperature of 108.0 to 110.0 F., the number of lymphoid areas was less in all chickens given heat treatments than in the controls. For the pancreas, the average numbers were 4.37 to 5.07, compared to 7.40 for a constant sample area (a significant difference). For the liver, average numbers were 3.05 to 3.63, compared to 4.04 (a nonsignificant difference).

These data support a previously developed hypothesis that ectopic lymphoid foci are a result of defense reactions by the body but are not ontogenetically normal structures of the bird, although commonly present.

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SUMMARIO IN INTERLINGUA

Le Influentia de Tractamento Thermic Super le Numero de Ectopic Focos Lymphoide in Gallinas

In unicrestate leghorns blanc, le temperatura rectal monta ab le nivello medie de 104,2 F un die post le covation ad un nivello de 107,6 F al etate de 29 dies. Le valores citate a varie laboratorios pro le octave die es inter le extremo de 104,2 F e 107,2 F.

Quando gallinettas de etates de inter 1 e 16 dies esseva subjicite a inter tres e novem tractamentos thermic, cata un consistente de un exposition durante 45 minutas a un temperatura externe de inter 108,0 e 110,0 F, le numero del areas lymphoide esseva reducite in omne casos in comparation con gallinettas de controlo sin tractamento thermic. In le pancreas, le numero medie esseva 4,37 a 5,07, comparate con 7,40 pro un constante area de observation. Iste differentia es statisticamente significative. Pro le hepate, le valores medie esseva 3,05 a 3,63, comparate con 4,04. Iste differentia non es significative.

Iste datos supporta un previemente disveloppate hypothese que ectopic focos lymphoide es le resultato de un reaction defensive del parte del corpore e que illos non es ontogeneticamente normal structuras del ave, ben que illos es communmente presente.

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Differentiation of Quail Bronchitis Virus and Infectious Bronchitis Virus by Heat Stability

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RECENT EVIDENCE [4] indicates that quail bronchitis virus (QBV) and chicken embryo lethal orphan (CELO) virus are the same agent. In this report, the term CELO will be used to indicate isolates originally designated by that name.[12-14] Quail bronchitis virus has been isolated from bobwhite quail in West Virginia [11] and Texas.[5] The CELO virus has been isolated from embryonating chicken eggs which were inoculated with unrelated materials [12-14] and from uninoculated embryonating chicken eggs intended for virus isolation and propagation work.[4] Reports on the characteristics of QBV (CELO)[4,5,11,13] show that the agent causes a respiratory disease in bobwhite quail; has infected chickens and turkeys without causing a recognized disease syndrome; does not agglutinate chicken red blood cells; and causes a typical mortality pattern, dwarfing, and gross lesions in the chicken embryo. The characteristics of infections bronchitis virus (IBV) are well described in the literature.[2,6-10] Hofstad [7] found that, of 60 IBV isolates diluted 10^{-2} in buffered tryptose broth containing 20 per cent horse serum, only one (No. 189) resisted 45 minutes of exposure to 56 C. The same isolate remained infective after 15 minutes at 56 C. in the same dilution without horse serum. Both IBV and QBV (CELO) have been differentiated by serum-neutralization tests but not by gross lesions in infected chicken embryos.[4,5,11,13]

Some diagnostic laboratories confirm tentative diagnoses of avian respiratory diseases by isolation of the agents in embryonating chicken eggs, but they are limited in the use of serum-neutralization tests. Under such circumstances, IBV has been identified on the basis of typical lesions and dwarfing in inoculated chicken embryos plus inability of the agents to agglutinate chicken red blood cells. The IBV and QBV agents cannot be differentiated readily on this basis. Both QBV and CELO have shown marked heat stability;[3,4,13] IBV is relatively heat labile.[7]

In preliminary work, two standard strains and one immunologically different strain of IBV were differentiated from QBV and CELO by heat-stability tests.[3,4] The purpose of the present study was to evaluate heat stability as a means of differentiating IBV and QBV.

MATERIALS AND METHODS

Five strains of QBV isolated from bobwhite quail (*Colinus virginianus*, Linné) were used in the heat stability trials. Strain QB 101 was isolated in 1956,* and has been characterized.[3-5] Strains QB 103, 104, 105, and 106 were isolated from cases of quail bronchitis in 1957.* Eighteen strains of IBV were tested for heat stability. Fifteen were those isolated from birds from field cases by the diagnostic laboratory during the 1958 calendar year.* Three strains, 93a, 189, and 581, were obtained from M. S. Hofstad, Veterinary Medicine Research Institute, Iowa State College, Ames. Strains 93a and 581 were relatively heat labile.[7] Strain 189 had been isolated from turkey serum and was the most heat resistant of 60 IBV isolates tested by Hofstad.[7] During the period when heat-stability trials were in progress for this study, viruses not in use were stored in chorioallantoic fluid (c.a.f.) at −10 C. to −20 C. Later, during the serum-neutralization tests, storage was at −20 C. to −50 C.

Infectious bronchitis (IB) antiserum was produced in chickens by inoculating them with the Massachusetts strain ** of IBV in c.a.f. from the second chicken embryo passage. This antiserum neutralized over 10^9 embryo lethal doses at the 50

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* Isolated at the Poultry Diagnostic Laboratory, School of Veterinary Medicine, Texas A. & M. College and Texas Agricultural Experiment Station, College Station.

** Received by the Virginia Agricultural Experiment Station from Dr. Henry Van Roekel, University of Massachusetts, Amherst, in material from the 400th passage in chickens.

per cent end point ($e.l.d._{50}$) of the Beaudette chicken embryo-adapted strain of IBV † but did not significantly neutralize CELO,‡ QB 101, nor an immunologically different[8,9] strain (Connecticut A5968) of IBV.§ The CELO antiserum was produced in rabbits by inoculation with the virus ‡ originally designated CELO. This antiserum neutralized more than 10^6 $e.l.d._{50}$ of CELO but did not significantly neutralize Beaudette IBV or Connecticut IBV. Previous experience has shown no significant difference between antiserums produced against a QBV strain originally designated CELO and those produced against QBV isolated from quail.[4] In general, the procedures outlined by Cunningham[1] were used in inoculation of chicken embryos via the allantoic cavity, harvesting of c.a.f., and in serum-neutralization (SN) tests.

The test for heat stability was designed for laboratories with limited equipment and supplies. Virus materials were held at 1 C. to 4 C. when thawed but not undergoing heat treatment. Viruses in c.a.f. were diluted 10^{-2} in nutrient broth (Difco) and 5-ml. aliquots were placed in two or more rubber-stoppered glass tubes.‖ The tubes were completely immersed in a water bath at 56 C. They were allowed two minutes to reach 56 C. before timing was begun and were agitated at least once during the period of heat treatment. The material was then rapidly cooled to 1 to 4 C. and inoculated into 9-, 10-, or 11-day-old embryonating chicken eggs. Sets of 5 embryos were used to determine viability of the viruses in nonheated material and in material held at each period of heat treatment. Each embryonated egg was given 0.2 ml. of inoculum in the allantoic cavity. The c.a.f. used in all comparative tests contained no antibiotics and was from a series of embryo passages with no heat treatment between passages.

A minimum of three trials at 56 C. for 15 minutes was made on each of the IBV strains. Definite dwarfing, as judged by candling, of at least 3 out of 5 embryos was the criterion for heat stability of the virus. No trial was considered valid unless the nonheated control material caused dwarfing in all 5 inoculated embryos. The IBV strains not inactivated by 15 minutes at 56 C. were tested at 15, 30, and 45 minutes in succeeding trials. Although the same criterion was used to judge heat stability in material held for 30 and 45 minutes, c.a.f. was harvested from any dwarfed embryos and, with and without heat treatment, was inoculated into additional embryos.

Each of the IBV strains had been passaged recently in chicken embryos until a definite dwarfing pattern was observed. Strain 93a was received as nineteenth passage material and was tested in c.a.f. from the 21st passage. The other 17 strains of IBV were tested in c.a.f. of the third to the tenth passage. Of 52 samples in this range, 43 (82.7%) were from the fifth to the seventh passage.

Each of the five strains of QBV was tested at periods of 45, 60, and 90 minutes at 56 C. The QB 101 strain was tested in diluted c.a.f. from the third embryo passage. The QB 103 and QB 105 strains were from the seventh passage, QB 104 was from the eighth, and QB 106 was from the ninth passage. The inoculation, harvesting, dilution, and transfer of IBV and QBV, respectively, were performed in separate rooms and by different personnel.

The three strains of IBV showing greatest resistance to heat, 189 (from Hofstad), E48254, and T48254 (Texas), were tested for neutralization by IB and CELO antiserums. The E48254 and T48254 strains were from the same case but were obtained from aqueous humor and from the trachea, respectively.

All heat-stability tests were conducted at College Station, Texas. Production of antiserums and SN tests were conducted at Blacksburg, Virginia.

RESULTS

Two strains of IBV (93a, 581) obtained from Hofstad and 12 of the 15 strains isolated by the diagnostic laboratory were completely inactivated in each of three trials by 15 minutes at 56 C. Another strain (E48252) isolated by the diagnostic laboratory was heat labile under the criterion set up for this study but did produce dwarfing in 1 of 5 embryos in one of three trials with diluted c.a.f. held at 56 C. for 15 minutes. In two serial passages from this embryo, all embryos inoculated with nonheated, diluted c.a.f. were dwarfed. After being held at 56 C. for 15 minutes, the same material failed to produce dwarfing.

Strain 189 IBV, received from Hofstad, and two strains (E48254 and T48254) isolated at the diagnostic laboratory remained stable after 15 minutes at 56 C. in three trials with each strain. Hofstad's report[7] on the relative heat stability of 189 was confirmed. In a fourth trial, 189 was again stable after 15 minutes but not after 30 or 45 minutes at 56 C.

In the first trial at 30 and 45 minutes, neither E48254 nor T48254 remained viable after those periods of heat treatment. In the second trial, using another sample of the same inoculum, E48254 produced dwarfing in 1 embryo and T48254 caused dwarfing in 2 of 5 embryos after being held at 56 C. for 30 minutes. The c.a.f. harvested from the dwarfed embryos and re-

† Obtained from Dr. D. E. Davis, Dr. Boney Laboratories, College Station, Texas.

‡ Obtained from Dr. V. J. Yates, University of Rhode Island, Kingston.

§ Obtained from Dr. R. E. Luginbuhl, University of Connecticut, Storrs.

‖ Vacutainers, No. 3200, manufactured by Becton-Dickinson & Co., Rutherford, N.J.

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passaged without heat treatment caused dwarfing in all inoculated embryos. In limited trials with successive passages of heat-treated E48254 and T48254 IBV, consistent dwarfing did not occur after 30 minutes of heat treatment.

With heat stability based upon definite dwarfing in at least 3 of 5 embryos inoculated with c.a.f. diluted 10^{-2} and held at 56 C. for 15 minutes, 15 of 18 IBV strains tested were heat labile. Strain 189, E48254, and T48254 were heat stable.

Serum-neutralization tests confirmed identification of 189, E48254, and T48254 as serologically standard strains of IBV. Massachusetts IBV antiserum neutralized 100,000 or more embryo infective doses of each of these three strains. They were not neutralized by CELO antiserum. Neither the IB nor the CELO antiserum used in this study significantly neutralized the immunologically different [9] Connecticut strain of IBV.

Each of the five strains of QBV produced dwarfing (and deaths) in embryos inoculated with c.a.f. diluted 10^{-2} and held at 56 C. for 45, 60, and 90 minutes. Compared to IBV, they were extremely heat stable.

Discussion

The marked heat stability of the QBV strains sharply differentiated them from the IBV strains. The 15-minute period arbitrarily selected as the criterion of heat stability for this study could serve to differentiate relatively heat-stable IBV and heat-labile IBV. A 30-minute period at 56 C. probably would be a better preliminary test for a diagnostic laboratory to use in differentiating QBV and IBV. The laboratory which is unable to use or must limit its use of the SN test could inoculate sets of 5 embryonating eggs with nonheated and heat-treated (30 min.), diluted c.a.f. containing the virus in question. Inactivation within 30 minutes at 56 C. would indicate that QBV was not present. A virus which remains viable after 30 minutes should be tested at 60- and 90-minute periods. Viability after 60 or more minutes at 56 C. would indicate the presence of QBV. Viruses surviving 30 minutes but not 60 minutes in two or more trials should be submitted to a laboratory which can carry out SN tests. Until more is known about the selective or adaptive action of heat on IBV populations, c.a.f. used in the heat-stability trials should not be from an embryo passage or series of passages in which the inoculum was exposed to heat.

This method for differentiating IBV and QBV was designed for simplicity and for minimum need of supplies and equipment. In research laboratories, a more critical approach, such as that used by Hofstad,[7] would be preferable. Although immunologically different IBV isolates have been reported,[8,9] they fall within the heat-stability range of immunologically standard IB isolates.[7,8] Increased confidence in heat stability as a means of rapidly differentiating IBV and QBV may result from trials using other dilutions, diluents, temperatures, and periods of heat treatment. Additional strains of the two agents should be tested. A disadvantage of the method in its present form is that it will not differentiate a mixture of IBV and QBV from QBV alone.

Summary

The heat stability of infectious bronchitis virus (IBV) and quail bronchitis virus (QBV) was compared, and 18 strains of IBV were differentiated from five strains of QBV by this method. None of the IBV strains consistently survived 30 minutes at 56 C. Identity of the three most heat-tolerant strains of IBV was confirmed by serum neutralization. All of the QBV strains survived 90 minutes of treatment at 56 C.

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SUMMARIO IN INTERLINGUA

Differentiation de Virus de Bronchitis de Colinos ab Virus de Bronchitis Infectiose per Thermostabilitate

Le thermostabilitate de virus de bronchitis infectiose e de virus de bronchitis de colinos esseva determinate. Le methodo esseva usate pro differentiar 18 racias de virus de bronchitis infectiose ab cinque racias de virus de bronchitis de colinos. Nulle del racias de virus de bronchitis infectiose superviveva uniformemente 30 minutas a 56 C. Le identitate del tres plus thermo-tolerante racias de virus de bronchitis infectiose esseva confirmate per neutralisation de sero. Omne le racias de virus de bronchitis de colinos superviveva 90 minutas de tractamento a 56 C.

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Allergy Studies on Cattle Vaccinated with Killed *Mycobacterium paratuberculosis*

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VACCINATION as a control measure against Johne's disease has received attention from a number of workers.[2-4,7] The vaccine consists of 5 to 30 mg. of living *Mycobacterium paratuberculosis* suspended either in a mixture of mineral oil and olive oil with powdered pumice stone added or in mineral oil alone. These products are injected subcutaneously. In England, vaccination has been confined to calves less than 30 days of age, since older animals might contract the disease prior to vaccination and evidence of the disease in such animals would confuse the results. According to Doyle,[2] subcutaneous inoculation with live culture did not produce infection in 5 animals that were examined at necropsy. There was a mass of field evidence confirming that subcutaneous inoculation with certain strains of living Johne's bacilli does not produce the disease. Vaccinated animals will give positive reactions to the intradermal johnin test, and they may also give false positive reactions to the tuberculin test. Therefore, the vaccine cannot be used in the United States without causing some interference with the tuberculosis eradication program. Sigurdsson[6] vaccinated thousands of lambs with 5 mg. of heat-killed *Myco. paratuberculosis* suspended in mineral oil. Results of his experiments indicate that vaccinated sheep develop resistance to Johne's disease. Since young cattle are very susceptible to infection, a vaccine that would protect young animals and not interfere with diagnostic test procedures for tuberculosis and paratuberculosis would be useful in the United States, even if the immunity were lost by the time they became adults.

The purposes of this experiment were: (1) to determine the period of time required for calves vaccinated with killed *Myco. paratuberculosis* to lose their sensitivity to tuberculin and johnin, and to find whether the sensitivity is lost more rapidly if the vaccination nodules are removed; (2) to determine if the size of the dose has any effect on the time required for a vaccinated animal to lose its sensitivity; (3) to determine if it is possible to distinguish between paratuberculosis and tuberculosis sensitization through comparative intradermal testing with tuberculin and johnin; and (4) to determine to what extent sensitization produced by *Myco. paratuberculosis* vaccine will mask a subsequent sensitization produced by *Mycobacterium tuberculosis* var. *bovis*.

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PROCEDURE

Sixteen male calves were purchased when 1 day old from herds known to be free of both tuberculosis and Johne's disease. All were negative when tested with intradermal tuberculin and johnin.

The calves were allotted to two equal groups by random selection. At approximately 3 weeks of age, each calf in group 1 was given an intradermal vaccination in the brisket with 0.1 ml. of a mineral oil suspension of 35 mg. per milliliter of killed *Myco. paratuberculosis*. Each calf in group 2 was vaccinated in the same manner, except that 0.3 ml. of material of the same strength was used. A nodule, about 5 cm. in diameter, formed at the vaccination site of each animal within one month after vaccination. One animal in group 2 died from an unknown cause three weeks after vaccination, leaving 7 animals in this group. Two months after vaccination, the nodules were surgically removed from 4 animals selected at random from both groups. The surviving animals in both groups were tested with 0.2-ml. doses of standard field johnin and tuberculin, given intradermally in both the cervical region and the caudal fold at intervals during a two-year period. They were disposed of after this time. Reactions were recorded on the basis of "pea" size of "P-reactions" since this is the standard method used by veterinarians in the field.[1] The P-1 reaction is $\frac{3}{16}$ inch thicker than the original skin thickness. The P-2 reaction is two times the size of P-1 and the P-3 reaction is three times the size.

The tests conducted on the caudal fold were made at approximately 90-day intervals. This interval was used to avoid the desensitizing effects of one test having an adverse effect on subsequent tests. Tests on different sites in the cervical region

were made at weekly intervals for the first nine weeks to determine the time required for vaccinal sensitization to reach its maximum. After maximum sensitivity had been reached, the interval between tests was increased. Twenty-two months after vaccination with *Myco. paratuberculosis*, each of 6 animals (3 with vaccination nodules and 3 with nodules removed) were given 1.5 ml. of a mineral oil suspension of 25 mg. per milliliter of heat-killed *Myco. tuberculosis* var. *bovis* subcutaneously.

At the termination of the experiment, all the animals were killed and examined. Smears of lymph glands from animals given only the *Myco. paratuberculosis* vaccine were treated with an acid-fast stain and examined microscopically.

RESULTS

Two weeks after vaccination, 8 of the calves had small reactions to both the johnin and the tuberculin tests. The largest reactions during the entire 20-month period were observed 40 days after vaccination.

In the cervical region, the johnin reactions varied in size from P-2 to P-5, and the tuberculin reactions varied from P-1 to P-3. In the caudal fold, the johnin reactions varied in size from P-1 to P-4, and the tuberculin reactions ranged in size from P-1 to P-2. There was no difference in the sensitivity of those animals with the vaccination nodules and those from which the vaccination nodules had been removed. The size of the vaccinal dose (0.1 ml. vs. 0.3 ml.) affected neither the degree nor the persistence of allergy produced. Comparison of 72 individual tests in both caudal folds showed that the johnin reactions were larger than the tuberculin reactions in 52 instances. Both reactions were of equal size in 16 instances, and the tuberculin reactions were larger in four instances.

Of 132 comparative tests on the cervical region, the johnin reactions were larger than the tuberculin reactions in 108 instances, both reactions were of equal size in 21 instances, and the tuberculin reactions were larger in three instances.

Twenty months after vaccination against Johne's disease, the caudal folds of 4 animals had lost all sensitivity to the tuberculin test. The arithmetical mean sizes of reactions to the intradermal johnin and tuberculin tests are shown (fig. 1). The mean of the tuberculin reactions was less than the mean of the johnin reactions during the 20 months these animals were under test. Both the johnin and tuberculin reactions in the cervical region were larger than those in the caudal folds.

The results of tests on the 6 vaccinated animals given killed *Myco. tuberculosis* var. *bovis* are shown (fig. 2). The average size of the tuberculin reactions was larger than the johnin reactions in animals given killed *Myco. tuberculosis* var. *bovis* in mineral oil. On a comparative basis, the tuberculin reactions were larger than the johnin reactions in the caudal folds of 3 animals and the reactions to both products were of equal size in 3. In the cervical region, the tuberculin reactions were larger than the johnin reactions in 5 animals and both reactions were of equal size in the sixth.

No gross abnormalities were found on postmortem examination, except in the vaccination nodules which consisted of fibrous tissue enclosing several small abscesses. Smears made from these abscesses

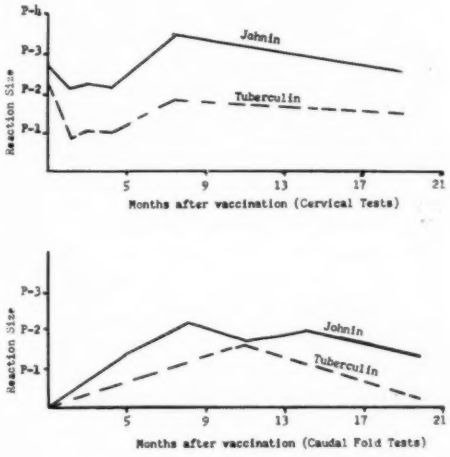

Fig. 1—A comparison of mean sizes of reactions to intradermal johnin and tuberculin tests in animals sensitized with killed Mycobacterium paratuberculosis.

disclosed small acid-fast bacilli, identical in morphology to those in the original vaccine. A few, scattered, acid-fast bacilli were found in a submaxillary gland of 1 animal and in an anterior cervical gland of another.

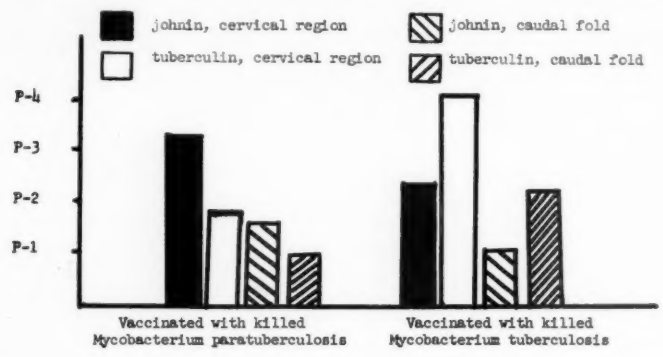

Fig. 2—The allergic response to tests on 6 calves. The calves were first vaccinated with killed Mycobacterium paratuberculosis. After 22 months, the same calves were vaccinated with killed Mycobacterium tuberculosis var. bovis. They were tested over a two-year period with caudal and cervical injections of johnin and tuberculin. The allergic reactions were recorded on the basis of "pea" size, shown on the ordinate.

DISCUSSION

Twenty months after vaccination against Johne's disease, sensitivity to the tuberculin test had disappeared in some animals and was very low in the others. If these animals had been officially tested at that time with tuberculin in the caudal folds, 8 of them would have been designated as negative. The remaining 7 animals had very small reactions which would not be classed as positive unless they were in a known tuberculosis-infected herd.[6] Removal of nodules at the site of vaccination did not reduce the sensitivity of animals to either tuberculin or johnin. The size of the vaccinal dose used in this test had little effect on the degree or persistence of allergy; however, if the larger dose had been ten times the quantity of the smaller dose, the differences would have been more pronounced.[5]

In this experiment, the johnin reactions were larger than the tuberculin reactions in about 75 per cent of the tests. The differences were more pronounced in comparative tests made on the cervical region than in those made on the caudal fold. When 6 of these animals were subsequently given injections of killed *Myco. tuberculosis* var. *bovis*, the tuberculin reactions became larger than the johnin reactions in most instances. Again, the differences were more pronounced in the cervical region than in the caudal fold.

These results confirm previous findings; *i.e.*, cattle vaccinated with *Myco. paratuberculosis* will react to the tuberculin. However, when johnin and tuberculin were injected simultaneously, the johnin reactions were larger than the tuberculin reactions in three fourths of the reacting animals. In those animals subsequently vaccinated with *Myco. tuberculosis* var. *bovis*, most of the tuberculin reactions were larger than johnin reactions.

SUMMARY

Eight calves were vaccinated intradermally with a 0.1-ml. dose of killed *Mycobacterium paratuberculosis* and 8 were given a 0.3-ml. dose. Nodules which developed at the vaccination site were removed from half of each group of animals. All were given johnin and tuberculin intradermically in both the cervical region and the caudal folds at intervals during a 22-month period. The results were as follows:

1) The size of the vaccinal dose or the removal of the nodule had very little effect on the degree of sensitivity or the rate at which it was lost. The sensitivity decreased in 20 months to a point where 8 of the animals had no reaction to the tuberculin test in the caudal folds.

2) The johnin reactions were larger than the tuberculin reactions in both the caudal folds and the cervical region in 160 tests out of a total of 204.

3) Six cattle vaccinated with *Myco. paratuberculosis* and subsequently vaccinated with killed *Mycobacterium tuberculosis* var. *bovis* had larger tuberculin than johnin reactions in three out of six instances in the caudal fold and five out of six in the cervical region.

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SUMMARIO IN INTERLINGUA

Studios de Allergia in Bestial Vaccinate con Morte Mycobacterium paratuberculosis

Octo vitellos esseva vaccinate per via intradermal con un dose de 0,1 ml de morte *Mycobacterium paratuberculosis*. Octo alteres recipeva 0,3 ml. Nodulos que se disveloppava al sito del vaccination esseva removite in un medietate del animales in ambe gruppos. Omnes recipeva johnina e tuberculina per via intradermal in le region cervical e le plicas caudal a intervallos durante un periodo de 22 menses. Le resultatos esseva le sequentes:

1. Le magnitude de dose vaccinal e le ablation del nodulos habeva pauchissime effecto super le grado del sensibilitate o super le rapiditate de su perdita. Le sensibilitate decresceva in 20 menses usque a un nivello ubi 8 del animales habeva nulle reaction al test de tuberculina in le plicas caudal.

2. In 160 ex un total de 204 tests, le reactiones a johnina esseva plus marcate que le reactiones a tuberculina, tanto in le plicas caudal como etiam in le region cervical.

3. Sex pecias de bestial vaccinate con *Myco. paratuberculosis* e subsequentemente con morte *Myco. tuberculosis* var. *bovis* habeva plus marcate reactiones a tuberculina que a johnina in tres ex sex essayos in le plicas caudal e in cinque ex sex essayos in le region cervical.

Experimental Bovine Coccidioidomycosis

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MANY THOUSANDS OF CATTLE in southwestern United States are infected with coccidioidomycosis each year. This information is furnished in published reports based on the observations of epidemiologists, pathologists, and veterinary meat inspectors, [1,3-5,7,8,12-14,16,17] as well as from a coccidioidin skin-test survey recently completed in Arizona.[8a] However, it has not been possible to associate clinical signs of the disease with the presence of coccidioidal lesions in the bovine.

In this study, further evidence of the benign nature of bovine coccidioidomycosis is presented.

MATERIALS AND METHODS

Three separate studies were conducted over a five-year period, during which 93 cattle were inoculated with sodium chloride suspensions of spores and mycelial particles of *Coccidioides immitis*. Tests for coccidioidin sensitivity were made with various lots of coccidioidin four days before experimental inoculation and at intervals following the inoculation. Serums for complement fixation and precipitin serology were collected before experimental inoculation and at varying intervals thereafter from some of the cattle used.

The Coccidioides fungus used for inoculum was originally isolated from a dog. Sabouraud's dextrose agar cultures were flooded with normal saline and the mycelial mat was scraped with an inoculating loop, after which the resulting suspension was agitated with sterile sand or mixed at 14,000 r.p.m. for one minute in a Sorvall Omni-mixer in an effort to obtain single spore fragments. On the basis of total spore counts made in a hemacytometer, a portion of the suspension was diluted and an estimate of viability was obtained by counting colonies after plating on Sabouraud's dextrose agar. Final dilution was made according to the viability of the suspension, so that 1 or 2 ml. would contain the number of viable particles to be given to each animal.

All of the cattle, except the controls, were inoculated by spraying the suspension of spores and mycelial particles into the trachea through a 14-gauge, 2-inch needle inserted between the tracheal rings posterior to the larynx.

The skin sensitivity tests were made by injecting 0.1-ml. amounts of the skin test agents intradermally and observing the size of the induration 96 hours later (fig. 1). Reactions 5 mm. or more in diameter were considered specific responses to the agents used. Coccidioidins used in the various tests were lots 58, 59, and 59-62, obtained from Dr. C. E. Smith of the University of California, and lots pooled A, 25A-1, 25A-2, and 26-A from the Arizona State Department of Health (ASDH). These coccidioidins were prepared by the technique described by Smith *et al.*,[15] except that the ASDH preparations were thoroughly agitated and the growth was submerged at monthly intervals.

Histoplasmin and haplomycin, used along with coccidioidin to check for cross reactions, were prepared and supplied by Dr. Furcolow and Dr. Menges, Public Health Service, Communicable Disease Center. The use of these agents has been described.[6, 9-11]

All complement fixation and precipitin serology was performed through the courtesy of Dr. C. E. Smith, with the facilities of the Armed Forces Epidemiological Board.

Material from lesions found at necropsy was cultured on Sabouraud's dextrose agar, both with and without cycloheximide and chloramphenicol.

Study A.—Study A was a preliminary trial involving 10 animals. During the experiment the animals were confined in roofed pens on a well-cultivated experimental farm near Tucson. Eight of the animals used were Hereford heifers, 6 months old, obtained from a southern Arizona desert range. The other 2 were bull calves from the Tucson area; 1 was a Guernsey, 1 week old, and the other a Holstein-Friesian, 3 weeks old. All animals, except the dairy calves, were checked for coccidioidin sensitivity before use, and serums from them were found to be free of complement-fixing or precipitin antibodies. The animals were obtained from endemic areas. However, they were used on the basis of the earlier observations that range-raised cattle were usually lesion-free at slaughter unless previously confined in a feedlot[12] and because the age of the dairy calves had provided little opportunity for natural exposure. The fallacy of these assumptions will be discussed later.

From the U.S. Public Health Service, Communicable Disease Center (Maddy), in cooperation with the Arizona State Department of Health, Phoenix (Snell), and the College of Agriculture, University of Arizona, Tucson (Reed and Trautman).

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The authors thank those of the Departments of Animal Science and Animal Pathology, University of Arizona, for their cooperation, and Mr. Ernest A. Crofford, Woodruff, Ariz., for providing the experimental animals.

Two of the Herefords were given 500,000 viable particles from a week-old culture; 2 were given 1 million particles from a culture 116 days old; and 2 were given 1.5 million particles from the same culture. The 2 dairy calves were inoculated with 2 million viable particles from a culture 6 months old. The 2 remaining Herefords were used as controls.

Three Herefords (2 principals and 1 control) were checked for coccidioidin sensitivity with undiluted and 1:10-diluted lot 59 coccidioidin in the cervical area before inoculation and two, three, five, seven, and 12 weeks after inoculation. Blood for complement fixation and precipitin serology was collected from these animals at the same intervals. Rectal temperatures were taken daily for the first 42 days after inoculation. These animals were kept for 130 days before slaughter. Lesions found at necropsy were cultured and histologic sections were made.

The other 5 Herefords (4 principals and 1 control) were checked for coccidioidin sensitivity using undiluted and 1:10 diluted lot 59-62 coccidioidin before inoculation and two, four, six, eight, and 12 weeks later. Blood for serologic work was taken from these animals before inoculation and two, three, four, six, eight, and 12 weeks after inoculation. Rectal temperatures were taken daily for the first 49 days after inoculation. Ninety-two days after inoculation, the 5 animals were slaughtered and the lesions found were cultured and sectioned.

The 2 dairy calves were checked for coccidioidin sensitivity with undiluted lot 59-62 coccidioidin three, six, and eight weeks after inoculation and for complement fixation and precipitin antibody formation on the day of inoculation and three, six, and eight weeks later. Rectal temperatures were not taken. After observation for 120 days, these animals were slaughtered and examined for lesions.

Study B.—In study B, 91 cattle were used. During the study, the cattle were kept in pens of a typical feedlot on a different experimental farm near Tucson where there was a considerable amount of dust. All were Hereford steers, 18 months old, obtained from a central Arizona mountain range at about 8,000 feet altitude, well outside the endemic area. Cattle were given 500,000 viable particles each from a culture 4 months old.

The 91 cattle used in this study were checked for skin sensitivity to lot D-2770 histoplasmin and lots 58 and 59-62 coccidioidin before use, after which 43 cattle were inoculated intratracheally with the particle suspension. No tests for development of complement-fixing or precipitin antibodies were made during the 90-day observation period, and rectal temperatures were not taken; however, since the cattle were on feeding trial, they were observed for general health, feed consumption, and rate of weight gain. At the end of the trial, control and inoculated animals were again checked for sensitivity to lots 58 and 59-62 coccidioidin and to lots 25A-1 and pooled A, after which they were slaughtered and examined for lesions. All skin-test agents used in this study were undiluted.

Fig. 1—The cutaneous lesion on the cervical area is a positive reaction to the coccidioidin test.

Study C.—Study C was a field experiment involving 65 animals maintained on pasture outside the area endemic for *C. immitis.* The steers and heifers (5 Hereford, 2 Angus, and 58 Holstein-Friesian) averaged 8 months of age and were being raised in northern Arizona (Woodruff) at a 5,200-ft. altitude.

Before experimental inoculation, all cattle were checked for sensitivity to lots 59-62 and 26A coccidioidin, lot D-2770 histoplasmin, and lot Phillips 4 antigen 16 haplomycin. No serologic tests were made and rectal temperatures were not taken. Twenty-five animals were given 1.4 million viable particles from a culture 3 months old, and 17 were given 700,000 particles from the same culture. The 42 inoculated and 23 control animals were observed for 90 days by their owner for signs of infection, after which tests for sensitivity to coccidioidin lots 59-62, 25 A-2, and 26A and to haplomycin were made. All skin-test agents were used undiluted. Cattle in this group remained in the possession of their owner and were not immediately slaughtered.

RESULTS

There were no clinically evident signs of illness in any of the 93 inoculated cattle in studies A, B, or C. Fever, a characteristic of coccidioidal infection in man and the dog, did not develop in study A animals. Rates of weight gain were equal in the control and inoculated animals; in the feedlot trial, feed conversion efficiency was the same in control and inoculated animals. The test animals slaughtered were found acceptable as human food.

Study A.—All coccidioidin tests were negative in the first 3 animals, except for the test at 12 weeks on 1 of the inoculated Herefords. In the second part of the study, 1 of the 4 inoculated animals gave positive coccidioidin reactions to the tests made six and 12 weeks after inoculation. Both of the dairy calves gave positive coccidioidin

reactions to the injections made three weeks after inoculation.

None of the serums collected contained complement-fixing antibodies.

Of the first 3 animals, 2 were negative for precipitins. In the other animal, serums drawn two and three weeks after infection gave 4+ (diagnostic) reactions in undiluted serums. Of the next 5 cattle, the control and 2 principals gave negative precipitin reactions. Serums from the third principal gave diagnostic reactions in both undiluted serums and the 1:10 dilution of serums collected two, four, six, and eight weeks after inoculation. In the fourth principal, a diagnostic level of precipitins appeared in undiluted serums three, four, and six weeks after inoculation and in the 1:10 dilution of serum collected three weeks after inoculation.

Necropsy revealed no grossly visible lesions in the control and in 1 principal of the first group. Two small pulmonary granulomas were found in the other principal, but the organism was not recovered in culture or found in tissue sections of the granulomas. The control and 3 principals in the second group had no grossly visible lesions, except in 2 principals that had developed coccidioidal abscesses under the skin where the trachea was perforated during inoculation. One of the 5 animals had extensive involvement of both lungs, with numerous small coccidioidal granulomas. *Coccidioides immitis* was recovered in cultures from lung and peritracheal lesions and was present in tissue sections. No grossly visible lesions were found in the other 2 animals used in this study.

Study B.—At the start of the study, the histoplasmin and coccidioidin tests were negative in all animals, except 1 which reacted to both coccidioidins used. After 90 days of observation, only 86 of the 91 animals could be definitely identified, due to loss of ear tags. Of the 40 identified principals, 32 gave positive reactions to lot 59–62 coccidioidin, 31 gave positive reactions to lot 58, 12 gave positive reactions to lot 25 A-1, and 7 gave positive reactions to lot pooled A. Ten of the 46 identified control animals gave positive reactions to both coccidioidin lots 59-62 and 58, 5 reacted to lot 25 A-1, and 1 reacted to pooled A. Whether inoculated or not, all animals that reacted to lots pooled A, 25 A-1, or 58 also reacted to lot 59–62.

At necropsy, 7 of the 10 skin-test positive animals in the control group had granulomas that were typical of natural infection; 3 had lesions in the bronchial and mediastinal lymph nodes only; 3 had lesions in the lungs and also in the bronchial or mediastinal lymph nodes, or both; and 1 had lesions in the submaxillary lymph nodes. Only 1 principal was found to have a lesion (a coccidioidal abscess) under the skin at the site of inoculation. *Coccidioides immitis* was identified in direct smears and by cultures from all lesions.

Study C.—Initial skin sensitivity tests were negative in all the animals. Only 63 of the 65 animals were available at the end of the observation period because 2 had died of posteastration septicemia not related to the experiment. At the end of the observation period, none of the animals were sensitive to haplomycin, all of the inoculated cattle were sensitive to the three coccidioidins used, and the controls were negative to the same agents.

DISCUSSION

Coccidioidal infection of cattle was accomplished experimentally by Giltner,[7] who found that subcutaneous inoculation of the fungus produced local infection of the skin and subcutaneous tissues but that intravenous inoculation resulted in rapidly fatal pulmonary disease. When Beck *et al.*[2] injected large doses of *C. immitis* into the flanks of 6-month-old steers, only local infection developed over a 10-week period. Similar local infection was observed in peritracheal tissue contaminated in the process of inoculating some of the cattle used in studies A and B. This observation served to confirm infectivity of the inoculum, which may have been questioned in view of the few thoracic lesions found at necropsy. No previous experimental inoculation of cattle with this fungus via the respiratory route has been reported, although this route is acknowledged as the most likely portal of entry for natural infection.

The results reported here were gathered over several years in the sequence presented. Knowledge acquired during early phases of the work and from investigations into other aspects of coccidioidal disease in animals points to obvious faults in the first efforts to determine the pathogenesis of bovine coccidioidomycosis. For instance, animals brought directly to slaughter houses

from southern desert ranges may not show coccidioidal lesions when killed, yet the desert affords ample opportunity for exposure. The probability that a calf will encounter infection within the first 6 months of life in the area from which the 8 Herefords used in study A were obtained is about 1:3, as indicated by coccidioidin sensitivity surveys in progress. Previous exposure and prompt recovery would lead to a high degree of resistance to challenge.

Results of this and other work in progress show that cattle infected with coccidioidomycosis produce few antibodies detectable by precipitin and complement-fixation tests, which would invalidate the serologic tests used in screening cattle for previous exposure. For this reason, serums were not collected in studies B and C.

Lot 59 coccidioidin, used in skin sensitivity checks of the first 3 Herefords (study A), was found to be less capable of evoking interpretable responses than coccidioidins used later, such as lot 59–62. Thus, an animal previously exposed but having borderline skin sensitivity may have been accepted as susceptible due to a poor or negative reaction to the coccidioidin employed. The sum of these findings in preliminary study A indicated that the only reliable assurance of susceptibility to coccidioidal infection lay in obtaining cattle from outside the endemic area.

It was learned that, for proper spore formation, cultures of *C. immitis* used as inoculum must be at least a month old. Suspensions of a week-old culture used to infect 2 animals in study A possibly contained far fewer actual spores than the viability count indicated, since nonsporulating mycelial fragments may have produced growth on the test plates.

Finally, though there was no choice in the test animals available for studies A and B, the thick, loose-skin characteristics of the neck region of Herefords was found to produce a less interpretable reaction to coccidioidin than occurs in dairy breeds.

Natural exposure potential in the course of trials A and B (held in the endemic area) was recognized before starting but was judged unimportant in the artificially infected cattle, since high numbers of fungus particles were used in the inoculums. Since the source of cattle in study B was a good guarantee of susceptibility through lack of previous exposure, control animals that reacted to the coccidioidin-sensitivity test or had lesions at necropsy were recognized as victims of natural infection. Other studies [8] have revealed that lesions of cattle with naturally acquired coccidioidomycosis often heal and are no longer grossly visible 30 to 60 days after they are acquired. Thus, it was no surprise that the lungs of the principals in study B had no grossly visible lesions when cattle were slaughtered 92 days after inoculation.

The results of study C were graphic. All inoculated animals became coccidioidin-sensitive, all controls remained negative, and there were no clinical signs of disease. These results, supported by the other trials and by the healthy appearance of many cattle found to be infected at slaughter, would seem to indicate that coccidioidomycosis, a common infection of the cattle in the endemic area, is a benign, asymptomatic, self-limiting process. Reliable evidence that cattle have been exposed to *C. immitis* may be obtained by a skin-sensitivity test with a sufficiently potent coccidioidin.

SUMMARY

Ninety-three cattle were each experimentally inoculated by the intratracheal route with 500,000 to 2 million viable particles of the saprophytic phase of *Coccidioides immitis*. No signs of illness were observed in 90 to 130 days. Precipitin antibody formation in inoculated animals was erratic. Complement-fixation antibodies were not detected. Serology did not afford a useful method for assaying progress of the disease or determining susceptibility. Most of the experimentally inoculated cattle developed skin sensitivity to coccidioidin, but not all preparations of coccidioidin employed proved capable of evoking reactions.

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SUMMARIO IN INTERLINGUA

Coccidioidomycosis Bovin Experimental

Novanta-tres pecias de bestial esseva inoculate experimentalmente per via intratracheal con doses de inter 500.000 e 2 milliones viabile particulas del phase saprophytic de *Coccidioides immitis*. Nulle signos de morbo esseva observate intra 90 a 130 dies. Le formation de anticorpore a precipitina in le inoculate animales esseva erratic. Anticorpore de fixation de complemento non esseva detegite. Le serologia non provideva utile methodos pro evalutar le progresso del morbo o pro determinar le susceptibilitate del animales. Le majoritate del inoculate animales disveloppava sensibilitate cutanee pro coccidioidina, sed non omne preparatos de coccidioidina usate se monstrava capace a evocar reactiones.

Studies on Infection and Immunity with the Cattle Lungworm, *Dictyocaulus viviparus* (Bloch). I. Infection in Laboratory Animals

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A SMALL laboratory animal that is susceptible to infection with the cattle lungworm, *Dictyocaulus viviparus* (Bloch), would facilitate preliminary evaluations of the effects of drugs or antigens in reducing or preventing infections with this parasite. *Dictyocaulus viviparus* has been recovered from the lungs of guinea pigs, rabbits, and mice.[2, 6, 11] Limited tests have shown that *D. viviparus* migrates to the lungs of these animals. However, the duration of the infection or resistance established has not been fully investigated. Therefore, the following experiments were conducted in an attempt to evaluate the effects and duration of lungworm infections in some of the common small laboratory animals.

MATERIALS AND METHODS

Host Specificity.—Lungworm larvae were recovered from the feces of infected calves and cultured to the infective stage by the procedures outlined by Swanson *et al.*[8] The third-stage larvae were administered to rabbits in doses of 4,000 to 60,000, and to guinea pigs in doses of 2,000 to 60,000, through a size 8 soft rubber catheter inserted into the stomach. Larvae were administered to hamsters in doses of 10,000 to 40,000; rats, 5,000 to 13,000; and mice, 3,000 to 23,000, through a needle inserted orally into the esophagus. Goats were given 10,000 larvae through an equine catheter passed through a mouth speculum into the rumen.

These experimental animals (principals) were killed at different periods after infection. The worms present in the lungs of the small animals were recovered as follows: The lungs were filled with 0.85 per cent sodium chloride solution, and the trachea was closed with a hemostatic forceps. The lungs then were sliced in thin sagittal sections, beginning at the distal ends of the diaphragmatic lobes and continuing upward toward the trachea. As the lung tissue was sliced, the NaCl solution in the air passages proximal to the cut flowed through a 20-mesh wire screen supported approximately half the distance from the bottom of a 250-ml. beaker. The lung material, suspended over this wire screen, was then covered with NaCl solution poured into the beaker. The container was covered and the preparation maintained at 35 C. for 15 to 18 hours. The lung sections were then lifted free of the NaCl solution and rinsed thoroughly with fresh solution. The worms in the sediment were counted in flat-bottomed culture dishes with a stereoscopic microscope. When red blood cells obscured the worms, an equal volume of 5 per cent acetic acid was added to hemolyze the cells and thereby clear the suspension. Worms were removed from the lungs of goats in a similar manner, using larger beakers.

Reactions of Guinea Pigs to Infection.—Fifteen male guinea pigs, weighing 481 to 666 Gm., were allotted to two groups, 10 in one and 5 in the other, by a stratified randomization technique in accordance with body weights. Sixty thousand living third-stage lungworm larvae were administered to each of the 10 principals in group 1 by stomach tube. The 5 guinea pigs in the second group were given no larvae and were maintained as normal controls under the same environmental conditions. The ration, consisting of commercial guinea pig meal * fed *ad libitum*, was supplemented twice weekly with fresh cabbage. All animals were weighed weekly. Feed consumption was measured daily.

Blood samples were obtained by cardiac puncture nine days prior to infection and 30, 45, and 129 days postinfection. The serum was separated within two hours after blood was obtained, and complement-fixing antibody determinations and paper electrophoretic studies of the serum proteins were conducted. Complement-fixation tests were performed in a manner similar to the "⅓ method" described by Kolmer *et al.*[5] Serial dilutions of positive serums were routinely prepared, ranging from 1:2 to 1:256. The complement reagent was prepared from desiccated guinea pig complement (Cappel) and titrated prior to use each day. The antigen, a 5.0 per cent suspension of lyophilized lungworms in NaCl solution containing 0.5 per cent phenol, was frozen and stored at −20 C. in 1-ml. quantities. From this finely ground stock suspen-

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* Purina chow, produced by Ralston Purina, St. Louis, Mo.

sion, the optimal dilution of 1:640 was prepared for use each day.

The criterion for a positive serum reaction was complete inhibition of cell hemolysis. The highest serum dilution at which this inhibition occurred was recorded as the antibody titer of the serum. Included in each test were a serum-control tube, an antigen control, a red blood cell hemolysis control, and a red blood cell control. A known positive calf serum having a titer of 1:32 was titrated each day to check the reproducibility of the test so that comparisons of titers from day to day would be valid.

The serum components were separated using the Spinco (Model R Durrum type) hanging strip paper electrophoresis apparatus. A veronal buffer solution with an ionic strength of 0.1 and a pH of 8.5 was employed and a constant current of 5 ma. effected the separation. The serum proteins were separated on Whatman No. 1 filter paper strips (Spinco). The strips were removed from the cell after 16 hours, dried for 30 minutes at 120 C., and the proteins were dyed with bromphenol blue. The dyed strips were scanned in a chromatographic strip reader.* The areas under the respective curves were calculated and the amount of each serum component was expressed as a percentage of the total serum protein.

Blood samples were taken from the ear veins of guinea pigs twice weekly prior to infection and every third day for 60 days following infection. Total red blood cell and leukocyte counts were made, and the differential leukocyte count was determined from blood smears stained with Wright's stain. Blood cell counts, complement-fixation antibody titers, electrophoretic data, and food consumption of infected versus noninfected animals were compared, utilizing an analysis of variance technique for comparing groups of unequal numbers.[3]

* Spinco Model R Analytrol and Integrator, manufactured by Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.

RESULTS

Host Specificity.—Lungworms were recovered from the lungs of hamsters five to seven days postinfection, from goats three to ten days postinfection, from rabbits three to 16 days postinfection, and from guinea pigs one to 38 days postinfection. Experiments involving infections in goats were not conducted beyond ten days. In the small laboratory animals, the recovered worms were sexually immature. The maximum lengths of worms recovered from the lungs of the various animals were: hamsters, 660 μ; mice, 390 μ; rabbits, 620 μ; goats, 1,500 μ; and guinea pigs, 6,450 μ. The mean numbers and lengths of worms recovered from these animals are indicated (table 1). The range in length of worms recovered from 1 calf, dying 19 days postinfection, is included for comparison. Rats were completely resistant to infection in these experiments. Using the length of worms recovered or the period of established infection as criteria, the guinea pig and goat were the most satisfactory hosts of the species exposed. Clinical signs observed in the guinea pig included anorexia, loss of weight, and coughing. The infections occasionally resulted in death.

Reactions of Guinea Pigs to Infection.—Four of 10 infected guinea pigs of group 1 died four to 38 days after infection. Worms were recovered from the animals that died on the fourth, 22nd, and 38th

TABLE 1—Mean Length (μ) and Mean Numbers of Dictyocaulus viviparus Recovered from Animals at Varying Intervals Postinfection

Days postinfection	Mice		Hamsters		Rabbits		Guinea pigs		Goats		Cattle	
	No. worms	Length	No. worms	Length	No. worms	Length	No. worms	Length	No. worms	Length	No. worms	Range in length
1	5	298	....	.....	....	.....	.....	.....	.....	.....	.....	.....
2	24	316	....	.....	....	.....	.....	.....	.....	.....	.....	.....
3	16	325	....	.....	25	365	308	351	90	378	.....	.....
4	6	350	....	.....	....	.....	398	395	.....	.....	.....	.....
5	3	355	12	320	3	378	179	410	.....	.....	.....	.....
6	3	360	....	.....	....	.....	414	391	135	580	.....	.....
7	0	.....	4	544	3	476	34	607	.....	.....	.....	.....
8	0	.....	....	.....	....	.....	89	783	.....	.....	.....	.....
9	6	327	0	.....	0	.....	73	1,054	.....	.....	.....	.....
10	0	.....	....	.....	....	.....	73	1,207	90	1,388	.....	.....
15	..	.....	0	.....	....	.....	2	3,276	.....	.....	.....	.....
16	0	.....	....	.....	1	323	1	2,700	.....	.....	.....	.....
19	..	.....	....	.....	....	.....	.....	.....	.....	.....	793	3,200–14,200

Number of worms recovered was calculated per 5,000 larvae administered.

days postinfection, but not from 1 dying 36 days postinfection. Although these animals were lost to further experimentation, they did serve to confirm the viability of the larvae used.

There was no significant difference between the groups of animals during the preinfection period with respect to feed consumption and total leukocytes or erythrocytes. It was found that cardiac puncture caused a diverse fluctuation in the differential blood leukocyte counts for five days; however, by the seventh day after puncture, the blood cell picture had returned to normal.

A significant lymphopenia and an increase in neutrophils occurred in the infected guinea pigs between the eighteenth and 33rd days (table 2). A significant rise in monocytes was evidenced by the third postinfection day and continued to be elevated significantly over the control animals for the entire 60 days of infection. Eosinophil and basophil cell counts did not change perceptibly during infection. No significant differences were observed in total leukocyte counts for the first 33 days of infection. The infected animals then developed a significant leukocytosis in comparison to the noninfected controls. No evidence of reduced numbers of erythrocytes was observed in the infected animals.

Food consumption was markedly depressed in the infected guinea pigs throughout the first six weeks of infection. The control animals consumed an average of 3.5 Gm. of food per day less than the principals prior to infection. However, during the first six weeks following infection, the controls consumed an average of 10 Gm. of feed per day more than the principals. This difference was reflected in greater weight gains of animals in the control group. The noninfected animals gained an average of 15.1 Gm. more than did the principals that survived the entire ten weeks of the experiment. The suppression in weight gain occurred shortly after infection and continued through the eighteenth day postinfection.

Electrophoretic analysis of the serums from these animals revealed an increase in the *gamma* fraction of the infected animals over that of the control group by the 30th postinfection day (table 3). There were no significant differences observed in the *alpha* and *beta* globulin fractions. The albumin fraction decreased in serums having elevated *gamma* globulin.

Two of the 10 principals had preinfection complement-fixation titers of 1:4 and 1:16, respectively. The 30-day postinfection titers of the principals had risen to a mean of 1:17 and thereafter rapidly decreased (table 3). The serums of the controls remained negative for complement-fixing antibody throughout the experiment.

TABLE 2—Total and Differential Leukocyte Counts in Guinea Pigs Following Infection with Dictyocaulus viviparus Larvae

Group	Period of infection (days)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Total leukocytes per cmm.of blood (thousands)
Infected	−11 to − 2	27.0 ± 1.7	68.0 ± 1.8	4.2 ± 0.3	12.5 ± 0.5
Control	−11 to − 2	27.6 ± 1.7	64.8 ± 2.0	5.3 ± 0.4	12.0 ± 0.6
Infected	+ 3 to +15	32.2 ± 2.0	60.8 ± 2.1	5.4 ± 0.5**	13.6 ± 0.5
Control	+ 3 to +15	33.3 ± 1.5	61.0 ± 2.1	3.0 ± 0.3	13.4 ± 0.6
Infected	+18 to +33	41.0 ± 2.2*	50.2 ± 2.2**	6.1 ± 0.5**	13.7 ± 0.5
Control	+18 to +33	33.0 ± 1.8	62.7 ± 1.9	2.8 ± 0.3	13.0 ± 0.5
Infected	+36 to +60	32.4 ± 1.5	61.5 ± 1.7	4.4 ± 0.4**	15.3 ± 0.5*
Control	+36 to +60	30.0 ± 1.1	64.4 ± 1.3	2.9 ± 0.2	13.5 ± 0.5

* Difference between means significant at the 5 per cent level. ** Difference between means significant at the 1 per cent level. All means recorded ± standard error.

DISCUSSION

The results of the experiments indicate that lungworms are rapidly eliminated from these hosts. When the logarithms of the numbers of worms recovered from the lungs of guinea pigs per 5,000 larvae administered were plotted as the ordinate and time after infection (in days) was plotted as the abscissa, a straight line having a correlation coefficient of −0.9469 was best fitted to the points. From this regression line, a biological half-life of lungworms in guinea pigs was calculated to be 1.35 days; *i.e.*, in each 1.35 days half of the existing worms were eliminated. Thus, when the

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criterion of worm burden decides the efficacy of the treatment, it is mandatory in controlled experiments involving comparisons between treated and untreated guinea pigs to have very exacting intervals between the time of infection, time of treatment, and time of slaughter.

TABLE 3—Complement-Fixing Antibody Titers and Percentage of Gamma Globulin in Serums of Infected and Noninfected Guinea Pigs

Group	Period of infection (days)	Complement-fixation titer	*Gamma* globulin (%)
Infected	−9*	1:2	8.1
Control	−9*	Neg.	8.5
Infected	+30	1:17	16.6**
Control	+30	Neg.	8.1
Infected	+45	1:5	14.8
Control	+45	Neg.	11.1
Infected	+129	Neg.	16.6**
Control	+129	Neg.	9.7

* Serums drawn nine days prior to infection with larvae. ** Difference between means is significant at the 5 per cent level.

Differences in length of worms and sexual development varied in the several hosts, which suggested that specific environmental conditions in the host may be necessary for optimal growth of the parasite. The growth rates of worms recovered from the laboratory animals recovering most rapidly appeared to be suppressed when compared with the growth rates of worms from goats, guinea pigs, and cattle (table 1).

It is evident that pronounced ill effects such as anorexia, weight loss, and death may result from infections of *D. viviparus* in guinea pigs. A blood cell disturbance was created by the experimental infection; however, in this respect, the reaction of the guinea pig to infection with this parasite was unlike that of the calf. Although an eosinophilia, as observed in calves, did not occur in guinea pigs, a significant monocytosis and neutrophilia existed during the postinfection period. This finding agrees with reports of some other parasitic infections in sheep and cattle.[1,4,7,9,10]

Complement-fixing antibody was present in demonstrable quantities for 45 days following infection. Complement-fixing antibody persists in calves for longer periods of time,[12] and may be due to the parasites remaining a longer time in the calf. The inflammatory reaction resulting from the inspiration of larvae and eggs into the alveoli of calves during the patent period of infection may also prolong antibody production.

Summary and Conclusions

1) Of the small laboratory animals (goats, rabbits, hamsters, rats, mice, and guinea pigs) exposed to *Dictyocaulus viviparus*, the guinea pig appeared to be the most susceptible.

2) Lungworms did not reach maturity in any of the small laboratory animals exposed to infection.

3) Infections in guinea pigs caused changes in the blood picture and such deleterious effects as anorexia, loss of weight, and death.

4) Complement-fixing antibody was demonstrated in the serums of infected guinea pigs 30 and 45 days postinfection, but none was observed 129 days postinfection.

5) The *gamma* globulin content of the serums of infected guinea pigs was significantly elevated by the thirtieth postinfection day, when compared with noninfected controls.

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SUMMARIO IN INTERLINGUA

Studios Relative al Infection per le Verme Pulmonar de Bestial, Dictyocaulus viviparus (Bloch) e al Immunitate Contra Ille Infection. I. Infection in Animales Laboratorial

1. Inter le minor animales laboratorial exponite a *Dictyocaulus viviparus* (i.e., capras, conilios, hamsters, rattos, muses, e porcos de India), le plus susceptibile pareva esser le porcos de India.

2. Le vermes pulmonar non attingeva maturitate in ulle del minor animales de laboratorio que esseva exponite al infection.

3. Le infection in le porcos de India causava alterationes in le sanguine e effectos adverse como anorexia, perdita de peso, e morte.

4. Anticorpore a fixation de complemento esseva demonstrate in le seros de inficite porcos de India 30 e 45 dies post le infection, sed nulle esseva observate 129 dies post le infection.

5. Le contento de globulina gamma in le sero del inficite porcos de India esseva significativemente elevate le trentesime die post le infection, in comparation con observationes in non-inficite animales de controlo.

Studies on Infection and Immunity with the Cattle Lungworm *Dictyocaulus viviparus* (Bloch) II. Reinfection Immunity in Guinea Pigs

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IT HAS BEEN demonstrated that the guinea pig may serve as an experimental host for the lungworm, *Dictyocaulus viviparus* (Bloch).[2-6] Complement-fixing antibody developed in infected guinea pigs, and serum *gamma* globulin increased during infection [4]; however, the antibody content of the serum decreased earlier after infection than in calves.[7] This finding suggested that reinfection immunity in guinea pigs, if produced, might be of short duration. The following experiments were designed to determine the presence and duration of reinfection immunity in guinea pigs.

MATERIALS AND METHODS

Thirty-nine male guinea pigs were allotted at random to nine groups. Eighteen of these animals, the principals (groups I, III, V, and VII), were given 20,000 to 60,000 infective third-stage [4] lungworm larvae in either one or two doses. The remaining 21 animals, maintained under similar environmental conditions, served as normal controls (groups II, IV, VI, and VIII). Blood samples (3 to 4 ml.) were collected two to nine days prior to inoculation, and also at the time of challenge and at necropsy. Complement-fixation tests were conducted on these serums as described by Wade *et al.*[4]

Reinfection of the principals with third-stage lungworm larvae was attempted 42 to 129 days after the initial inoculation. Thirteen of the 21 control animals were inoculated with larvae from the same cultures on the same days as the principals. The animals were killed 10 to 16 days after challenge. The lungworms were recovered, counted, and measured as previously described.[4] Schultz-Dale tests [1] were performed on the ilia of guinea pigs in groups I, II, III, and IV, and on the 8 remaining noninfected guinea pigs (group IX). Immediately after obtaining the last blood sample, the ileum of each was excised and washed in warm aerated Tyrode's solution. The terminal 4 cm. was quickly transferred to a Palmer muscle warmer and maintained in aerated Tyrode's solution at 37 C. for 15 minutes. At the end of this time, the muscle usually had recovered sufficiently to proceed with the test. The antigen, consisting of 0.2 ml. of a 1:10 suspension of ground lyophilized lungworms in Tyrode's solution, was added to the tissue bath and the muscle response was recorded on a smoked kymograph drum. When the muscle had relaxed to the original state, the system was drained and replaced with fresh Tyrode's solution. Five minutes later, antigen again was added to the bath. After the muscle tone had returned to the normal base-line level, 0.2 ml. of histamine solution, containing 2 μg. of histamine per milliliter, was introduced into the bath. The response of the muscle to the antigen was calculated as the percentage of the histamine response.

RESULTS

No lungworms were recovered from 5 of the 6 principals of group I, reinfected 129 days after initial inoculation and killed 15 days postchallenge. One lungworm was recovered from the sixth animal. The 4 control animals of group II, necropsied 15 days after initial inoculation, were all found to harbor lungworms. The numbers of lungworms recovered from the lungs of these 4 animals were 9, 19, 18, and 25, respectively (table 1). The worm burdens of animals challenged 129 days after initial inoculation and killed ten days after challenge also were found to be considerably less than those of the 2 control animals. The lungs of reinfected principals (group III) yielded 4, 8, 10, and 29 worms; the controls of group IV, initially inoculated ten days prior to necropsy, yielded 123 and 475 worms. The lungs of the previously exposed principals of group V yielded 5 and 15 worms; those of the initially inoculated controls of group VI yielded 22 and 27 worms. Three of the 6 previously inoculated principals of group VII were free of lungworms at necropsy and 3, 2, and 5 worms were recovered from 3 other animals of this same group. From the lungs of ani-

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TABLE 1—Effect of Initial Exposure on the Production of Resistance to Reinfection of Guinea Pigs with Dictyocaulus viviparus

Group No.	No. animals necropsied	Initial dose (No. of larvae)	Challenge interval (No. days postinoculation)	Challenge dose (No. of larvae)	Lungworms			
					No. recovered		Length	
					Mean	Range	Mean (mm.)	Range (mm.)
I	6	60,000	129	60,000	0.2	0–1	2.3	2.3
II	4	0		60,000	17.7*	9–25	3.2	0.1–6.5
III	4	20,000	129	30,000	12.7	4–29	0.8	0.4–1.2
IV	2	0		30,000	299.0	123–475	1.1**	0.5–2.1
V	2	20,000	84	60,000	10.0	5–15	....	.....
VI	2	0		60,000	24.5	22–27	....	.....
VII	6	44,500†	42	20,000	1.7	0–5	1.4	0.7–1.9
VIII	5	0		20,000	43.4*	27–77	1.5	0.6–2.1

Animals in groups I, II, V, and VI were necropsied 15 to 16 days after challenge. Animals in groups III, IV, VII, and VIII were necropsied ten days after challenge.
* Difference between means is highly significant. ** Difference between means is significant at the 5 per cent level. † Administered as two doses, given one week apart.

mals in control group VIII, killed ten days after initial inoculation, 25, 35, 38, 42, and 77 worms were recovered.

The lengths of the undistorted and unbroken worms recovered from animals necropsied ten to 16 days postinoculation are tabulated (table 1). In every case, the average lengths of the worms recovered from the challenged guinea pigs were shorter than those recovered from the animals initially inoculated. This difference in length was statistically significant in groups III and IV.

The complement-fixing antibody titers of animals initially given 60,000 larvae increased from zero prior to infection to an average of 1:28 by 15 days postinoculation. However, reinfection 129 days after initial inoculation caused the formation of demonstrable complement-fixing antibody in only 1 of the 6 animals challenged. Ten days postinoculation, complement-fixing antibody titers in the 2 control guinea pigs of group IV rose to an average of 1:12; whereas, only 1 of 4 reinfected animals of group III had complement-fixing antibody in demonstrable quantities (*i.e.*, 1:2).

The Schultz-Dale tests revealed a pronounced sensitivity of the ilia of initially inoculated and reinfected guinea pigs to lungworm antigens when compared with the reaction of the ilia removed from the normal noninfected guinea pigs (table 2). A nonspecific reaction occurred in the ilia of noninfected animals. This reaction was an increase in peristaltic movements rather than the single tetanic contraction observed in the ilia of initially inoculated and reinfected animals. This response was not related to an antigen-antibody reaction, as evidenced by the fact that the characteristic desensitization of the muscle did not occur when the ilia of these normal animals were exposed to this antigen.

DISCUSSION

It appears that antibody is formed rapidly in the guinea pig after infection with lungworm larvae. This antibody, which may be demonstrated ten to 15 days postinoculation in higher titer than found in calves,[7] probably is produced in response to the rapid migration of larvae through the lymphatics and into the lungs of guinea pigs.

The absence of complement-fixing antibody in the serums of challenged guinea pigs was not expected. Possibly the antibody measured by this method is an antibody produced in response to worms residing in the lungs. Therefore, with fewer worms residing in the lungs of challenged animals, there was little or no antigenic stimulation and, consequently, less antibody was formed. However, the protective mechanism responsible for preventing the development of worms may not be identical with the complement-fixing antibody measured in this experiment. Thus the larvae may

TABLE 2—Results of Schultz-Dale Test Performed on the Ilia of Guinea Pigs Infected with Lungworm Larvae

Group	No. of animals	Mean response of ilia to antigen (cm.)	Mean response of ilia to histamine (cm.)	Mean per cent of histamine response (cm.)
Reinfected (groups I and III)	6	4.10	0.88	465.9
Initial infection (groups II and IV)	4	2.14	0.49	436.7
Controls (group IX)	8	1.26	2.76	45.6

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have been destroyed by this protective mechanism before they developed or migrated sufficiently to stimulate the formation of complement-fixing antibody. Although complement-fixing antibody titers of the serums were generally negative, reinfection immunity was demonstrated 129 days after the initial dose of larvae was given. The smaller size of the worms recovered from the reinfected animals suggests that development of these parasites was inhibited.

In these respects, the guinea pig seems to be analogous to the calf. In order to utilize the guinea pig in preliminary evaluations of antigen activity in stimulating active immunity, these findings are of extreme importance. If little or no reinfection immunity could be demonstrated, it would be pointless to attempt to establish immunity by the injection of killed antigen.

The Schultz-Dale test [1] demonstrated that antibody was present in the tissues of challenged animals. This finding lends support to the hypothesis that at least a portion of the reinfection immunity is due to specific antibody. This tissue-phase antibody, located in the ilium, may render this organ a major site of reinfection immunity. Further studies will be necessary in order to determine the exact point at which the migration of larvae is blocked in the guinea pig.

SUMMARY AND CONCLUSIONS

1) A specific antibody, present in guinea pigs infected and challenged with *Dictyocaulus viviparus* larvae, was demonstrated by the Schultz-Dale test.

2) Complement-fixing antibody was demonstrated in guinea pigs ten and 15 days after initial inoculation with lungworm larvae. However, this was not observed in 8 of 10 reinfected guinea pigs in these tests.

3) Increased resistance to infection with *D. viviparus* was produced in guinea pigs as a result of a prior infection.

4) The increased resistance to infection was manifested by the occurrence of smaller and fewer worms in the lungs of reinfected animals at necropsy.

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SUMMARIO IN INTERLINGUA

Studios Relative al Infection per le Verme Pulmonar de Bestial, Dictyocaulus viviparus (Bloch) e al Immunitate Contra Ille Infection. II. Immunitate a Re-Infection in Porcos de India

1. Un specific anticorpore esseva demonstrate per medio del test de Schultz-Dale in porcos de India post infection e provocation subsequente con larvas de *Dictyocaulus viviparus*.

2. Anticorpore a fixation de complemento esseva demonstrate in porcos de India dece e 15 dies post le inoculation initial con larvas del mentionate verme pulmonar. Tamen, isto non esseva observate in 8 ex 10 re-inficite porcos de India in iste tests.

3. Plus forte resistentia contra le infection con *D. viviparus* esseva producite in porcos de India como resultato de un previe infection.

4. Le augmento del resistentia contra le infection esseva manifeste in le occurrentia de plus miere e minus numerose vermes in le pulmones de reinficite animales quando illos esseva necropsiate.

Infectious Bovine Keratoconjunctivitis. I. Etiology

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AN INFECTIOUS KERATITIS of cattle was first described in 1888, in Nebraska.[5] Since that time, there have been numerous reports of its occurrence in this country [1,3,4,14,22,24-27,36] and in several foreign countries.[6-11,17-20]

Infectious bovine keratoconjunctivitis (IBK) has become recognized as one of the more costly diseases of the cattle industry. Due to its widespread incidence and economic importance, considerable research has been done on the disease. In spite of this, a clear understanding of the condition has not been gained.

The bacterium, *Moraxella bovis*, has been incriminated as the etiologic agent by several investigators.[3,4,19,22,24-26,35,37] Other workers consider a Rickettsial agent to be the cause.[10,12] There are still others who suggest that the causative agent has not been identified or that there might be a dual relationship between different organisms to produce the disease.

The disease process itself and many of its basic aspects have not been adequately investigated. In fact, there are few reports in the literature of such investigations.

This report is the first of a series concerning investigations on IBK and the organism, *Mor. bovis*. The purpose of this report is to further clarify the etiologic role of *Mor. bovis* in IBK.

REVIEW OF LITERATURE

Billings,[5] in 1888, reported keratitis in cattle in Nebraska and described a short, thin bacillus with rounded ends which was found in stained corneal sections. The isolated organism did not reproduce the disease when inoculated into healthy eyes.

Penberthy,[22] in 1897, recorded four epizootics of keratitis, the first of which occurred in 1892 in England. He was able to transmit the disease by placing exudate from infected eyes into the eyes of 2 healthy cattle. One of 2 healthy calves placed in contact with diseased animals developed the condition. This was the first report indicating the infectious character of the disease, but no attempts were made to culture the causative agent.

The first evidence of an organism resembling *Mor. bovis* was reported by Mitter[20] in 1915. He identified a diplobacillus identical to the diplobacillus of Morax[21] and Axenfeld[2] from an epizootic of keratitis in cattle in Bengal.

Bacillus pyogenes was incriminated as the cause of keratitis in Holland in 1917 by Poels.[23] He quoted Schimmel, who indicated that rays from the sun may play an important role in the disease. Poels stated that the eye secretions were worthless from an etiologic standpoint, so he cultured scrapings from the affected corneas. These scrapings were stained and bacilli characteristic of *B. pyogenes* were found. Two species of micrococci were also found. The author considered the former to be the etiologic agent and the latter secondary invaders. Symbiosis, *in vitro* and *in vivo*, between the organisms isolated was considered. In order to reproduce the condition, Poels injected pure cultures of *B. pyogenes* beneath the epithelial layer of the cornea.

In 1919, Allen[1] reported an epizootic of infectious keratitis in cattle in Quebec, Canada. Direct smears demonstrated a short, thick, gram-negative diplobacillus. The discharges from infected eyes were collected and instilled into the eyes of 2 healthy animals. Typical infections resulted. Allen attempted to isolate the causative organism on agar, but failed. Then, using Loefflers' blood serum, he isolated an organism closely resembling a diplobacillus. It was pleomorphic and conformed to several of the characteristics described by Morax[21] and Axenfeld.[2]

In Ohio, Jones and Little[25-26] isolated a diplobacillus from 24 cattle with cases of acute ophthalmia. They were able to reproduce the disease by instilling a few drops of a pure culture of the organism into the conjunctival sac of normal cattle.

Cases of ophthalmia in cattle were described by Canham[9] in the Union of South Africa in 1923. Attempts to reproduce the disease by the transfer of ocular discharges were unsuccessful. Normal animals did not develop the disease when tied head-to-head and side-by-side with infected animals. A nematode of the genus Thelazia was found in some of the affected eyes.

The finding of Rickettsia-like bodies in conjunctival scrapings of infected eyes of cattle was reported by Cole[10] in 1936.

Magens,[28] in 1936 stated, "It is quite true that it [keratitis] should be regarded primarily as a secondary symptom of a number of febrile diseases. . . . In spite of this, however, observation of many herds forcibly brought me to be-

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lieve that infectious keratitis of ruminants may well become an etiologic entity.'' Both pneumococci and diplococci were demonstrated by Magens.

Carpano [9] described an epizootic of ophthalmia in cattle in 1938 and stated that the disease was of a contagious nature.

Contagious keratitis in a herd of 70 cattle in England was reported by Craig and Ratter [11] in 1940. Although experimental transmission attempts were unsuccessful, the history of the epizootic suggested the spread of some infection.

Farley [13] unsuccessfully attempted to reproduce the disease with filtrates of eye secretions from affected animals. The immunity of animals used was later challenged with unfiltered eye secretions, and 70 per cent became infected.

No single specific cause of keratitis in beef animals in the United States had been determined by 1943 according to Kingman *et al.*[27]

Baldwin,[3] in 1945, isolated *Mor. bovis* from cattle with cases of bovine infectious keratitis in the states of Ohio, Montana, and California. The bacterium was isolated from 83 per cent of the cattle. This investigator indicated that *Mor.* bovis was the probable cause of infectious keratitis.

Reid and Anigstein [30] investigated keratoconjunctivitis along the Gulf Coast of Texas in 1945. These authors indicated the contagious nature of the disease. Direct examination of smears from the conjunctivae of animals affected with the acute form of the disease revealed bacilli which usually appeared as diplobacilli. Some of the smears revealed numerous small coccal and diplococcal bodies within the cytoplasm of epithelial cells. Attempts to isolate the Rickettsia-like bodies were negative. Later, Reid and Anigstein [30] were able to isolate *Mor. bovis* from animals with cases of keratoconjunctivitis by using blood agar. The instillation of pure cultures of *Mor. bovis* produced the disease in susceptible animals. These writers stated, ''These results entitle us to the conclusion that the cultivated bacillus is the specific agent of infectious keratoconjunctivitis in cattle.''

Blakemore [6] investigated conjunctivitis of sheep and cattle in Great Britain in 1947. He demonstrated cell inclusion bodies in naturally infected cattle. Reproduction of the disease in a mild form was demonstrated by the instillation of lacrimal secretions from infected animals into normal eyes. Cell inclusions were found early in the course of the disease, but they disappeared shortly after signs of the disease were seen. However, the cell inclusions were again demonstrated when sodium hydroxide was dropped into eyes of recovered animals to produce an irritation.

Instillation of pure cultures of *Mor. bovis* into the eyes of cattle without producing the disease was reported by Farley [15] in 1950. Eight out of 29 of the same animals developed keratitis when they were exposed to eye secretions from infected cattle. *Moraxella bovis, Staphylococcus aureus* (both orange and white colonies), *Escherichia coli* and streptococci were isolated from cattle affected with the disease.[16] Farley [15,16] further indicated that *Mor. bovis* was not the primary factor in the production of keratitis of cattle. He considered it only a secondary invader.

Pook [24] reported 33 cases of conjunctivitis and keratitis in a group of 54 crossbred Holstein-Friesian calves. The conjunctivae of several were curetted and stained. Inclusion bodies were seen in the epithelial cells.

A description of keratitis, occurring in cattle of the New Forest area of England in the summer of 1951, was reported by Hall-Patch.[21] New Forest disease was the name given to the condition by this author. He indicated that the cause was uncertain.

Barner [4] reported on a comprehensive study of keratoconjunctivitis and *Mor. bovis*. *Moraxella bovis* was isolated from 92 of 95 acute cases. The organism was isolated from 3 of 5 animals when the nasal secretions were cultured. Cultures of *Mor. bovis* were transferred seven times on proteose peptone agar enriched with bovine blood. These cultures were then used to inoculate 4 normal calves intraocularly. All 4 developed the disease. Neither Pleuropneumonia-like organisms nor Rickettsia-like organisms were demonstrated in conjunctival scrapings of 36 normal and 23 diseased cattle. Barner concluded that keratitis was caused by *Mor. bovis*.

Jackson [24] isolated *Mor. bovis* from affected eyes. He reported that the disease was reproduced with cultures of the bacterium in cattle, sheep, and 1 goat. Melanin particles were demonstrated in the conjunctival epithelial cells from diseased cattle. These resembled rickettsiae in some respects.

Keratitis in cattle with corneal ulceration was reportedly due to the presence of Thelazia spp. according to Mitchell and Hughes [29] in England in 1953.

Infectious ophthalmia of cattle in Australia was recorded by Gallagher [19] in 1954. *Moraxella bovis* was isolated from animals with the disease. Melanin granules, but no Rickettsia, were found in stained conjunctival cells. The isolated cultures were capable of reproducing the disease in cattle. The author indicated that some epizootics in Australia may be due to other causes, but that it was probable that a great majority of them were caused by *Mor. bovis*.

The isolation of *Mor. bovis* from cattle in England was not successful, according to Formston.[18]

A Rickettsia was isolated from the aqueous humor of infected cattle in Germany by Dietz and Voigt [12] in 1956. A keratitis was reproduced in calves by the inoculation of the Rickettsia.

Hoffman [23] isolated *Mor. bovis* but no Rickettsia from cattle with cases of keratitis in South America in 1957.

Raghavachari and Reddy,[25] in 1957, described an epizootic of IBK in India. These authors isolated *Mor. bovis* from affected animals and indicated that other epizootics had been encountered from which the same organism had been isolated.

PROCEDURE

To further determine the role of *Mor. bovis* in the disease, routine attempts were made to isolate the organism from cattle in herds involved in epizootics of typical IBK. Lacrimal fluids, nasal secretions, and aqueous humor were cultured. Epizootics in 15 herds were studied (table 1).

Eyes showing typical early signs and lesions, such as marked lacrimation and photophobia accompanied by ulceration of the affected corneas, were selected for culture.

Lacrimal secretions were collected by introducing a sterile, dry, cotton swab into the conjunctival sac. The swab was rotated around the conjunctival sac and over the cornea, then streaked directly on Bordet Gengou (BG) agar plates containing 5 per cent citrated bovine blood and 2 per cent glycerin.

The cotton swabs were then placed in tubes containing 4 ml. of sterile nutrient broth, rotated several times, and withdrawn. Streptomycin and penicillin were added to the broth at rates of 100 mg. and 40,000 units, respectively. Following the addition of antibiotics, the material was allowed to remain at room temperature for 30 minutes. It was then held at approximately −23 C. until inoculated into embryonating chicken eggs.

The aqueous humor was obtained by puncturing the eyeball with a 19-gauge needle attached to a 5-ml. syringe and aspirating approximately 0.5 ml. The site of the puncture was at the lateral canthus approximately at the limbus. The aqueous humor was plated on BG, placed in broth, and treated as previously described for the lacrimal fluids.

Nasal secretions were cultured from 3 animals with typical signs of the disease. This material was collected on sterile swabs and plated on BG.

All cultures were incubated for 24 hours at 37 C. The plates were then observed for the presence of hemolytic colonies. Colonies that were convex, grayish white, entire, circular, and translucent were subcultured on BG; inoculated into dextrose, maltose, lactose, sucrose, mannitol, and litmus milk; and stained by a modification of Gram's method.

Colonies were considered to be *Mor. bovis* when they showed hemolysis on BG, did not produce acid or gas in carbohydrates, slowly peptonized litmus milk in six to ten days, and stained gram-negative with a typical diplobacillary appearance.

Colonies were considered to be of the smooth type when they were convex with circular edges. As the colonies became flat with undulate edges, they were called rough.

Cultures were designated smooth (S) or rough (R) according to the predominant colony type when cultured on BG. In those instances in which the S and R colonies were approximately equal in number, the designation SR was given to the culture.

Injections with 0.2 ml. of each sample of both lacrimal fluids and aqueous humor from epizootics A, B, C, D, and E were made into the allantoic cavities of 5 10-day chicken embryos. The inoculated eggs were candled daily. If no changes were observed in the embryos six days postinoculation, amnioallantoic fluid was harvested from 2 eggs, plated on nutrient agar, and passed serially in another set of eggs. Five serial passages were made. If no embryo changes were detected by this time, passage was discontinued.

The pathogenicity of *Mor. bovis* for calves was determined by three methods. The first was by directly inoculating a susceptible animal with material from an infected 1. The inoculums were obtained by swabbing the infected eyes with sterile cotton swabs and then inoculating the susceptible animal by rotating the exudate-impregnated swabs in the conjunctival sac and over the cornea. Material was inoculated onto BG plates to determine the presence of *Mor. bovis* and the colony types.

The second method utilized pure cultures of *Mor. bovis* obtained by picking isolated colonies. These cultures were serially passaged on BG. When ready for use as inoculum, approximately 2 ml. of sterile sodium chloride solution was poured onto the agar surface of each plate. A sterile inoculating wire was used to dislodge the colonies, and the bacterial suspension was removed with a sterile syringe.

Inoculation of the eyes was accomplished by introducing cotton swabs saturated with the harvested *Mor. bovis* cultures beneath the eyelids of the animal. The swab was also moved over the conjunctiva and cornea. The lower lid was then held away from the eyeball and some of the culture material was poured into the lower conjunctival sac. The eyelids were then gently massaged.

The third method used lyophilized cultures which had been isolated from epizootic J. The cultures had been lyophilized and maintained at −23 C. for six months prior to use.

The lyophilized cultures were resuspended in sodium chloride solution and inoculated onto BG. The BG plates were then placed in a 37 C. incubator for 18 to 24 hours. After incubation, the cultures were harvested by the method described for agar transfers or by removing the growth with a dry, sterile, cotton swab. The eyes were then inoculated as already described or the swabs were placed directly into the animal's eyes without suspending the growth in sodium chloride solution.

Seven transmission trials were made. These are described in the order that they are given (table 3).

In trial 1, a Hereford-Jersey crossbred calf was inoculated directly with material from the eyes of an animal in epizootic H. The affected calf had two small ulcers on the right cornea.

In trial 2, 3 Jersey calves were used. Material from the eyes of these calves was cultured for *Mor. bovis* before they were inoculated; all samples were negative. The third agar passage of a *Mor. bovis* culture isolated from epizootic I was used as inoculum.

In trial 3, material from the eyes of the 6 calves was cultured and found negative for *Mor. bovis*. The calves were then inoculated with a sodium chloride suspension of *Mor. bovis* isolated from epizootics E and F. These animals were inoculated on four different days during a five-day period.

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TABLE 1—Isolations of Moraxella bovis Made from Cattle Involved in Infectious Bovine Keratoconjunctivitis Epizootics

Epizootic	No. affected	No. animals tested	No. animals positive	Colony type*
A	40	14	13	S
B	3	1	1	SR
C	1	1	1	SR
D	13	12	11	S
E	1	1	1	R
F	1	1	1	S
G	1	1	1	SR
H	2	2	2	S
I	20	12	10	S
J	12	1	1	S
K	16	11	11	S
L	13	7	6	S
M	52	6	5	SR
N	19	19	19	S
O	8	4	3	S
15	202	93	86	

* S = smooth; R = rough; SR = approximately 50–50.

On the first and second days, the third agar passage of one strain was used. On the third and fifth days, the fourth and fifth agar passages of the second strain were used.

In trial 4, 20 calves in epizootic D were naturally exposed to the disease over a period of approximately one month. Seven did not develop signs, while 13 did. The 7 unaffected calves were later inoculated by direct swab from a calf with a relatively mild case. One week later, the unaffected calves were inoculated with a sodium chloride suspension of five different strains of *Mor. bovis* ranging from the first to the fifteenth agar passage.

TABLE 2—Results of Attempts to Isolate Agents other than Moraxella bovis in the Chicken Embryo

Epizootic	No. cultured	No. embryo passages	Results
A	9	5	Neg.
B	1	5	Neg.
C	1	5	Neg.
D	2	5	Neg.
E	1	5	Neg.

In trial 5, a Jersey and a Jersey-Brahman crossbred were inoculated with a lyophilized culture of *Mor. bovis* isolated from epizootic J. Both animals' eyes were negative for *Mor. bovis* prior to inoculation. One lyophilized tube was resuspended in NaCl solution and plated on BG. The growth of *Mor. bovis* obtained was taken from the plates by a dry swab and inoculated directly into the eyes.

Eighteen out of 20 calves in trial 6 were raised in a screened, isolation barn out of contact with other cattle. *Moraxella bovis* was not isolated from any of these eyes when material from them was cultured prior to inoculation.

Inoculation was by agar cultures and a modified direct method. The agar cultures were from two sources, both of which originated from lyophilized cultures from epizootic J. One was a resuspended lyophilized culture inoculated onto BG, while the other was a culture isolated from 1 of the calves in trial 5. Both cultures were the first agar passage following resuspension or isolation.

The modified direct method used a sodium chloride suspension of lacrimal secretions from the same calf in trial 5. Secretions were obtained by repeatedly swabbing the infected eyes and suspending the collected material in 4 ml. of NaCl solution, which diluted the collected material to an unknown degree.

The right eye of each calf was inoculated with the agar cultures, while the left was inoculated with the suspended lacrimal secretions.

In trial 7, 16 Hereford and 3 Hereford-Angus crossbred calves were exposed twice with cultures isolated from infected calves in trial 6. The first inoculation was with a NaCl suspension of one isolate, while the second was composed of five different isolates. The two exposures were made one week apart.

RESULTS

The results of the isolation attempts from lacrimal fluids from animals in natural epizootics of IBK are given (table 1). A total of 66 isolations were made from 73 animals in 15 herds. The principal colony type for each isolate is also given (table 1).

Moraxella bovis was isolated from nasal secretions of 3 animals. All attempts to isolate the organism from the aqueous humor, however, gave negative results.

At no time was there embryo dwarfing, death, or other changes in the embryo passages. The results are summarized (table 2).

The results of the transmission trials and the method of inoculation are given (table 3). A total of 43 of 59 animals inoculated developed the disease. The condition was produced by all methods of inoculation employed.

DISCUSSION

Agents other than *Mor. bovis* were not isolated from cattle with clinical cases of

TABLE 3—Ability of Moraxella bovis Cultures to Produce Infectious Bovine Keratoconjunctivitis when Inoculated Ocularly in Calves

Trial No.	Method of inoculation	No. inoculated	No. positive
1	Direct	1	1
2	Agar passage	3	2
3	Agar passage	6	3
4*	Agar passage and direct	7	0
5	Lyophilized	2	1
6	Agar passage and modified direct	20	18
7	Agar passage	20	18
		59	43

* These animals had been exposed naturally for a long period of time.

IBK under the conditions of these experiments. This supports recent unconfirmed reports of similar experiments done elsewhere.

Moraxella bovis was isolated from 90 per cent of the lacrimal secretions cultured, while this organism was not isolated from the aqueous humor of any of the animals. The predominant colony type isolated was in the smooth phase.

The isolation of *Mor. bovis* from nasal secretions of infected animals emphasizes the importance of expelled nasal secretions in the spread of the disease.

The disease was reproduced in calves by three methods of inoculation. Lyophilization or a limited number of serial agar passages did not result in loss of pathogenicity.

Trial 4 results were included in figuring the percentage infection even though these animals had been exposed naturally and had been refractive to the naturally occurring disease. When this group is excluded, the percentage infection from experimental exposure changes from 72 to 82 per cent.

In trial 6, the right eyes were inoculated with agar-grown cultures while the left eyes were inoculated with a NaCl suspension of infected lacrimal secretions. Fourteen of the calves developed the disease in the right eye only or in the right eye sooner than in the left. In 4 of the calves, the left eye became infected first.

If the disease were caused by a viral or Rickettsial agent, it would seem probable that the amount of virus present would be greater in the NaCl-suspended conjunctival swabbings than in the agar passage bacterial cultures. The former was used immediately after collection while the latter had been incubated at 37 C. on BG. If the disease were caused by a bacterial organism, however, the bacterial cultures would have a higher concentration of organisms than the lacrimal suspension. The bacterial cultures should therefore produce the disease to a greater degree. This is what happened in trial 6.

SUMMARY

Moraxella bovis was isolated from lacrimal secretions of 90 per cent of the cattle with clinical cases of infectious bovine keratoconjunctivitis. It was not isolated from the aqueous humor of any infected animals. Other agents were not isolated from the lacrimal secretions and aqueous humor under the conditions of these experiments.

Infectious bovine keratoconjunctivitis was experimentally produced in 43 of 59 calves inoculated by three methods. Koch's postulates in respect to *Mor. bovis* were fulfilled.

This work further strengthens the belief that *Mor. bovis* is the etiologic agent in infectious bovine keratoconjunctivitis.

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SUMMARIO IN INTERLINGUA

Infectiose Keratoconjunctivitis Bovin. I. Etiologia

Moraxella bovis esseva isolate ab secretiones lacrimal de 90 pro cento del bestial con formas clinic de infectiose keratoconjunctivitis bovin. Le organismo non esseva isolate ab le humor aquose de ulle del inficite animales. Nulle altere agentes esseva isolate ab le secretiones lacrimal e le humor aquose sub le conditiones del presente investigation.

Infectiose keratoconjunctivitis bovin esseva producite experimentalmente in 42 de 59 vitellos inoculate per tres differente methodos. Le postulatos de Koch con respecto a *Mor. bovis* esseva confirmate.

Le studio corrobora additionalmente le these que *Mor. bovis* es le agente etiologic in infectiose keratoconjunctivitis bovin.

The Effects of Testicular Biopsy on Spermatogenesis and Testicular Cytology in the Bull

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CONSIDERABLE controversy exists concerning the practicability of testicular biopsy in the bull. The underlying cause of the disagreement is whether the degenerative changes resulting from the technique overshadow its usefulness as an experimental or diagnostic aid. In order for a testicular biopsy technique to prove satisfactory and practical in clinical and experimental work, it appears that at least two criteria must be met. First, an adequate sample of the testicular tissue must be removed intact in order that fixation and cytologic studies can be made. Second, the operator must be reasonably certain that the removal of such a section of tissue from the testicle will not disturb spermatogenesis and normal testicular function to any marked degree. The first of these criteria appears to have been met by use of the modified 12-gauge Vim-Silverman needle technique described by McDonald and Hudson.[12] The second criterion has not been satisfactorily answered, according to the literature, because the work of Erb *et al.*,[5] Byers,[3] Sykes *et al.*,[15] Hill and Gassner,[7] Barker,[1] Roberts,[13] Ullner,[16] Leidl,[10] and Knudsen [8] are not in agreement and long-term studies have not been made to determine the effects of needle biopsy.

The purpose of this study was to determine the effects of needle biopsy on spermatogenesis by semen examination, and on testicular cytology by biopsy during the experiment and at slaughter. This study concerns 4 bulls studied during a four-month period following biopsy and 4 other bulls studied during a two and one half-month period one year following biopsy.

MATERIALS AND METHODS

Two groups of 4 Hereford range bulls were assembled on the basis of uniformity and absence of detectable disease. These bulls averaged 2½ years of age at the beginning of the experiment. Insofar as was known, they had not been used for breeding purposes or discarded for infertility. The bulls were maintained in a small exercise lot and fed a standard ration of prairie hay and a protein-vitamin-mineral supplement at a rate to provide for an average rate of growth.

The design of the experiments is shown (tables 1, 2). The first experiment involved a five-week, semen-collection control period followed by bilateral testicular biopsies; then a seven-week semen-collection period followed by second bilateral testicular biopsies; and, finally, a seven-week semen-collection period before slaughter and collection of testicular samples for histologic studies. The results of this experiment indicated that a longer postbiopsy period should be studied. Therefore, the second, or long-term, experiment on 4 bulls involved a five-week, semen-collection control period, followed by bilateral testicular biopsies. After a one-year rest, a semen-collection period of ten weeks preceded slaughter and testicular sectioning.

During the short-term study and during the prebiopsy period of the long-term study, facilities were such that electroejaculation had to be used in order to obtain the semen samples. During the postbiopsy period of the long-term study, facilities were available so that a "teaser" cow, breeding chute, and artificial vagina could be used to collect the semen samples. The semen samples were obtained at approximately four- to seven-day intervals by the use of an electroejaculator.[4] The quantity of the ejaculum was somewhat dependent on the operator of the electroejaculator, but an attempt was made to allow clearance of the tract by seminal fluid before semen collection. In the short-term study, 42 per cent of the samples were second ejaculums obtained after approximately a ten-minute rest period. During the long-term study when electroejaculation was used during the prebiopsy period, second ejaculums comprised 24 per cent of the samples. With the use of the artificial vagina in the postbiopsy period of the long-term study, second ejaculums comprised 20 per cent of the samples.

During use of the artificial vagina, the bull was momentarily restrained in the breeding chute to provide a teasing period before mounting the teaser cow. Since these were range bulls that were not halter broken, an adequate teasing period was difficult to provide. Immediately upon collection of the semen sample, the ejaculum was measured to the closest 0.25 ml. and the motility rating

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TABLE 1—Short-Term Effects of Biopsy on Spermatogenesis of 4 Bulls

	Prebiopsy period (12-12-55 to 1-11-56)					Between-biopsy period (1-20-56 to 3-6-56)					Postbiopsy period (3-29-56 to 5-10-56)				
	No.*	Mean	s.d.**	C†	Range	No.*	Mean	s.d.**	C†	Range	No.*	Mean	s.d.**	C†	Range
Volume of ejaculum (ml.)	55	8.18	3.14	38	2.0–15.0	57	8.83	3.22	36	4.5–17.0	42	9.17	3.13	34	5.5–18.0
Spermatozoa/ml. semen ($x10^3$)	55	380.73	303.4	80	10.0–1,030.0	57	459.12	325.8	71	50.0–1,270.0	42	504.04	454.9	90	100.0–1,350.0
Spermatozoa/ ejaculum ($x10^6$)	55	2,891.0	2,575.0	89	110.0–8,900.0	57	3,642.7	2,180.8	60	325.0–8,255.0	42	4,530.4	2,957.3	65	700.0–9,450.0
Motility rating	55	2.47	.....	....	0.0–5.0	57	3.21	.....	....	0.0–5.0	42	4.05	.....	....	2.0–5.0
Abnormal forms (%)	55	16.27	.....	....	2.0–64.0	57	20.21	.....	....	3.0–84.0	42	13.66	.....	....	2.0–37.0

* No. = number of observations. ** s.d. = standard deviation. †C = coefficient of variation.
The first bilateral biopsies were performed 1-12-56, the second were performed 3-14-56. Bulls were slaughtered 5-15-56, when testicular sections and radiographs were taken.

tabulated as 0 to 5, with 5 being the highest motility assigned. The spermatozoa count per milliliter was made with a hemocytometer and the eosin stain technique described by Smith and Mayer.[14] The per cent of abnormal forms was calculated from at least 100 observations, with abnormalities consisting of tailless form, double or piriform heads, curved or crooked tails, and protoplasmic droplets. The number of spermatozoa per ejaculum was the product of volume times the calculated spermatozoa count per milliliter of semen.

Testicular biopsy samples were taken bilaterally according to the technique of McDonald and Hudson.[12] Tissue sections were taken at slaughter from the interior, the periphery, and from the site of previous biopsy when identifiable. Postslaughter radiographs were taken of all testicles in order to identify further testicular alteration.

RESULTS AND DISCUSSION

SHORT-TERM EFFECTS OF BIOPSY ON SPERMATOGENESIS

The data (table 1) indicate that a depression of spermatogenesis did not occur in these bulls after two bilateral biopsies made two months apart. This judgment was based upon semen volume, spermatozoa per milliliter of semen, total spermatozoa per ejaculum, motility rating, and percentage of abnormal forms during the period of observation. Volume and, to some extent, total count depended upon the operator of the electroejaculator. The increase in spermatozoa per milliliter and total spermatozoa per ejaculum after biopsies may be assigned to the expected increase during maturation of these young bulls or to other unknown causes. These parameters of semen quality during the final postbiopsy period meet the requirements of Laing[9] for the classification of "potentially highly fertile bulls." The large standard deviations, coefficients of variation, and wide range of values indicate a large variation between and within bulls, as observed by other workers.[9] During the postbiopsy period, the coefficient of variation for spermatozoa per milliliter of semen was considerably larger (C = 43) than observed by Bishop *et al.*,[2] whereas the coefficient of variation for spermatozoa per ejaculum was similar to that observed by Bishop *et al.* (C = 56).[2] The apparent increase in motility during the experimental periods is also a subjective criterion which should receive only minor consideration, since the motility during all periods

appeared to be within normal limits. The per cent of abnormal forms appeared to be within normal limits.

Leidl [10] observed marked deterioration in semen quality for several months after open biopsy. Although the studies reported herein continued only four months after biopsy, there was no deterioration of semen quality. Apparently the difference between the two studies involved the biopsy technique and amount of tissue removed. Leidl [10] removed a section 6 by 5 by 3 mm.

Gross observation of the testicles at slaughter indicated no abnormalities except small puncture sites or minor adhesions of the tunica albuginea to the tunica vaginalis at the biopsy sites in two of eight testicles. The adhesions were not of sufficient magnitude to interfere with testicular mobility or circulation of blood of the testicle. Sections were taken from the interior, periphery, and biopsy sites in order to determine if there was histologic alteration of the germinal epithelium. The parenchymae of the testicles contained cores of calcification corresponding to the biopsy punctures. Histologic study of these biopsy sites revealed that cores of calcification, approximately 3 mm. in diameter, penetrated the testicles to the depth of all biopsies. Obstruction of the seminiferous tubules occurred throughout the calcification site, but adjacent seminiferous tubules appeared to be functioning normally and spermatogenesis was interferred with only at the local site of biopsy. Radiographs of the excised testicles likewise revealed plugs of calcification at the sites of biopsy (fig. 1). Histologic examination of sections taken at slaughter revealed no alteration except as described at the immediate biopsy site.

LONG-TERM EFFECTS OF BIOPSY ON SPERMATOGENESIS

Electroejaculation was used to obtain semen samples during the prebiopsy period but, during the year after biopsy, facilities became available for use of an artificial vagina. It was felt that more usable data would be obtained during the postbiopsy period by use of the artificial vagina.

It is difficult to compare the data from the two periods because of the different means of obtaining semen samples. According to the data, the bulls in the prebiopsy period produced semen average in quality for young, untested, unselected, Hereford bulls. Greatest attention should be directed to the postbiopsy period, during which samples of semen were obtained by the artificial vagina one year after biopsy. These data indicate that semen volume, spermatozoa per milliliter of semen, and total spermatozoa per ejaculum meet the requirements of Laing [9] for "potentially highly fertile bulls." Semen volume was larger than the 4.82 ml. reported by Lindley *et al.*[11] for 31 Hereford bulls in Oklahoma during a seven-year period. These investigators recorded a lower volume in winter which, if excluded from their data, would cause the results of the two experiments to agree more closely. Total spermatozoa per ejaculum from bulls in the present study averaged 30 per cent higher than that of 7 dairy bulls 2 years old, in Oklahoma.[17] Motility rating and abnormal forms, although subjective criteria, were also within normal limits.

As observed in the short-term study, the large standard deviations, coefficients of variation, and wide range of values for all parameters during both pre- and postbiopsy periods indicate wide variation between and within bulls. The coefficients of variation

TABLE 2—Long-Term Effects of Biopsy on Spermatogenesis of 4 Bulls

	Prebiopsy period (4-12-56 to 5-17-56)					One year postbiopsy period (5-7-57 to 7-23-57)				
	No.*	Mean	s.d.**	C†	Range	No.*	Mean	s.d.**	C†	Range
Volume of ejaculum (ml.)	39	9.28	3.15	34	1.5–17.0	55	6.08	2.55	42	2.5–16.0
Spermatozoa/ml. semen (x10⁶)	39	274.6	246.7	90	40.0–1,140.0	55	507.5	447.4	88	20.0–1,680.0
Spermatozoa/ ejaculum (x10⁶)	39	2,445.8	2,206.3	90	330.0–9,975.0	55	3,045.3	2,870.8	94	70.0–10,725.0
Motility rating	39	3.62	.....	....	1.0–5.0	55	3.15	.....	....	0.0–5.0
Abnormal forms (%)	39	13.87	.....	....	1.0–43.0	55	28.05	.....	....	7.0–48.0

* No. = number of observations. ** s.d. = standard deviation. †C = coefficient of variation.
Bilateral biopsies were performed 6-15-56; bulls were slaughtered and testicular sections and radiographs were made 7-26-57.

Fig. 1—Radiograph of excised testicle (short-term study) showing localized area of calcification corresponding to sites of biopsy punctures made two and four months previously. Radiographs of testicles 13 months postbiopsy did not show calcification sites.

observed herein are considerably larger than those reported by Bishop *et al.*[2] This may reflect the fact that these bulls were unselected for fertility and semen production, such as regularly occurs in artificial insemination centers.

Gross observation of the testicles at slaughter indicated no abnormalities except adhesions of the tunica albuginia to the tunica vaginalis at the biopsy sites in two of eight testicles. Motility of the testicles and circulation of blood did not appear to be altered. Sections were taken from the interior, periphery, and biopsy sites in order to determine if there was histologic alteration of the germinal epithelium (fig. 2). Particular attention was paid to the biopsy site, as calcification had occurred in the short-term study. There was no calcification present at the biopsy site in the bulls in the long-term study, as evidenced by histologic sections and testicular radiographs. Apparently, resolution and resorption of the mineral deposits had occurred. Likewise, there was no detectable alteration of the seminiferous tubules at the supposed site of biopsy, such as found after open biopsy by Gassner and Hill.[6] It appears from these observations that the calcification and tubule damage at the penetration site of the biopsy needle is transitory and that within a year repair processes resolve such alteration.

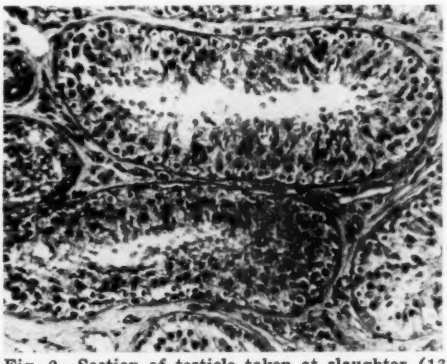

Fig. 2—Section of testicle taken at slaughter (13 months postbiopsy) showing normal spermatogenesis. H & E stain; original magnification x 75.

SUMMARY

A study was made of the effects that testicular biopsy with a 12-gauge Vim-Silverman needle may have on spermatogenesis and testicular cytology in the bull. The criteria of evaluation were: semen volume, spermatozoa per milliliter, spermatozoa per ejaculum, motility and abnormal forms of spermatozoa, and histologic study of biopsy and slaughter sections of the testicles. The study was divided in two parts: one concerned approximately six-week periods before, between, and after biopsies in 4 bulls; the other concerned a five-week period before biopsies and a ten-week period one year after biopsies in 4 bulls.

Based on the data collected, the biopsy procedure did not appear to affect spermatogenesis materially since, according to the criteria of the study, postbiopsy semen samples were within normal limits for such experimental bulls. Histologic studies of testicular sections taken at slaughter indicated that a transient calcification and alteration of the biopsy puncture site was present approximately two months after biopsy, but resolved approximately one year after biopsy. Some biopsy punctures resulted in mild adhesions of the testicular tunics without interference of testicular function. Although cytologic studies indicated that testicular biopsy did not adversely affect spermatogenesis in these bulls, no tests were made of the fertilizing capacity of semen from the biopsied bulls.

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SUMMARIO IN INTERLINGUA

Le Effectos de Biopsia Testicular Super le Spermatogenese e le Cytologia Testicular in le Tauro

Esseva studiate le possibile effectos resultante ab biopsia testicular con un agulia Vim-Silverman, calibre 12, in le spermatogenese e le cytologia testicular del tauro. Le criterios del evalutation esseva: Volumine del semine, numero de spermatozoos per millilitro, numero de spermatozoos per ejaculation, motilitate e anormalitate de forma del spermatozoos, e observationes histologic in sectiones bioptic e cadaverie de tissu testicular. Le studio esseva dividite in duo partes: Le prime colligeva datos durante periodos de approximativemente sex septimanas ante, inter, e post biopsias in 4 tauros. Le altere colligeva datos durante un periodo de cinque septimanas ante biopsias e durante un periodo de dece septimanas un anno post le biopsias in 4 tauros.

A judicar per le datos colligite, le manipulation del biopsia non pareva afficer le spermatogenese substantialmente, proque sub le criterios del presente studio, le specimens de semine postbioptic remaneva intra le limites del valores normal pro tal tauros experimental. Studios histologic de sectiones testicular obtenite al abattition del tauros indicava que un transiente calcification e alteration del sito del punction bioptic esseva presente approximativemente duo menses post le intervention sed se resolveva in le curso de approximativemente un anno. In un certe numero de casos, le punction bioptic resultava in leve grados de adhesion del tunica testicular sin interferentia in le function testicular. Ben que studios cytologic indicava que le biopsia testicular non afficeva adversemente le spermatogenese in iste tauros, nulle tests esseva facite con respecto al capacitate fertilisatori del semine ab le biopsiate tauros.

The 12-Gauge Vim-Silverman Needle for Testicular Biopsy of the Bull

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A CONSISTENT METHOD of performing a satisfactory testicular biopsy of the bull is needed. Such a technique should find wide application in clinical medicine as a means of differentiating the obstructive from the unobstructive form of azoospermia, determining the severity of the pathologic processes of oligospermia, classifying hypogonadic states accurately, and measuring the effects of therapeusis. Experimental studies of cattle would be enhanced by such a technique.

Testicular biopsy in the bull is not new, but previous workers are in disagreement regarding the effects of biopsy. Byers [2] reported testicular degeneration in some bulls and apparent success in others following a combination "open-punch" technique. Sykes *et al.*,[11] Hill and Gassner,[4] and Barker [1] described numerous retrograde changes following "open" biopsy. Leidl [6] employed the "open" technique of Ulner [12] and observed that degenerative changes in spermatogenesis followed the operation. Roberts [10] found depressed spermatogenesis in 2 bulls and normal spermatogenesis in 8 bulls by using the "open" method. Histologic alteration was observed at the site of biopsy in the 9 bulls slaughtered. McEntee [8] did not recommend use of the "open" method. Barker [1] and Knudsen [5] were optimistic about the use of the 14-gauge needle for testicular biopsy.

The experiment reported herein involved the screening of existing techniques for testicular biopsy in man and animals to find or develop a consistent method of obtaining an adequate sample for histologic study from the bull without adversely affecting testicular function.

PROCEDURE AND RESULTS

Three mature and 3 immature healthy Hereford bulls were used in the screening trials. The standard Vim-Silverman split needle (14-gauge) was used in the technique described by Barker [1] and Knudsen.[5] In general, this technique proved fairly successful on the immature bulls. Cross sections of approximately 13 seminiferous tubules were obtained per biopsy sample in 11 trials, but tubular separation and distortion were marked. Samples of testicular tissue from the mature bulls were unsatisfactory because the tubules separated instead of remaining intact as a "plug" for fixation. This separation may occur because of the small diameter of the plug or because of a decrease in the relative amount of supporting tissue after puberty.

An aspiration technique was tried next. The procedure and site were the same as described in the preceding trials except that, after removal of the trocar from the cannula, a negative pressure was applied with a 5-ml. syringe. The negative pressure was maintained while the cannula was withdrawn. The stringy, cloud-like masses of tissue obtained were impossible to fix with Bouin's solution.[9]

It was decided to try next the Charney method of sampling.[3] This is the method most widely used in testicular biopsy of man. It is accomplished by incising the tunica vaginalis communis, nicking the tunica albuginea testis, and extruding a bead of parenchyma by gentle pressure on the testicle. The bead of tissue is excised and placed in Bouin's solution. An attempt to use this exact technique in the bull was unsuccessful. Although the original nick in the tunica albuginea was enlarged to ¼ inch, no parenchyma could be extruded. Two additional bulls were subsequently sampled by using a ¼-inch incision and somewhat satisfactory samples were obtained. Hemorrhage was more copious than in any of the needle trials but no scrotal hematomas resulted. Testicles excised 43 days after biopsy had large diffuse areas of fibrosis extending deep into the testicle from the site of each biopsy, similar to the findings of Hill and Gassner,[4] Sykes *et al.*,[11] and Barker.[1]

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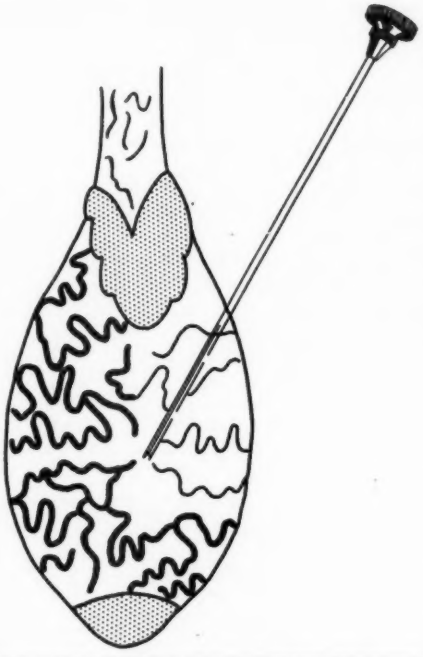

Fig. 1—Position of biopsy needle during biopsy in relation to epididymis and testicular axes.

Since the Vim-Silverman needle had been moderately successful in the young bull, the next trials were designed to extend this method. A larger instrument, a 12-gauge outer-cannula Vim-Silverman split needle,* was obtained by special order. Satisfactory samples of testicular tissue were obtained by a procedure similar to that employed with the 14-gauge needle. The bull was restrained in a squeeze chute with the hindleg on the operator's side tightly secured. The biopsy site was anesthetized by a pudendal nerve block or a posterior epidural block plus local infiltration of the skin. The testicle was clipped, disinfected, and firmly held at the bottom of the scrotum with the skin tightly drawn over the dorsolateral quadrant. The testicle was examined to make certain that the caput epididymis was not over the proposed site of biopsy. A sharp-pointed scalpel was thrust through the skin and tunics of the dorsolateral quadrant of the testicle and withdrawn, producing a ⅛-inch incision. With the testicle still firmly held with one hand, the trocar-cannula was inserted approximately ½ inch toward the center of the testicle by the operator (fig. 1), and then held by an assistant. The cannula was withdrawn by the operator. While the assistant held the trocar in a fixed position, the operator inserted the split needle (1 inch longer than the trocar) to its full length through the trocar, thereby cutting a "plug" of parenchyma. The trocar was then pushed over the split needle while the needle was held firmly. The trocar and needle were rotated at least 360 degrees and withdrawn. The split needle was withdrawn from the trocar and immersed in Bouin's fixative solution. After several minutes of fixation, the tissue could be teased from the needle as a plug. Partial fixation of the tissue while in the needle seemed to be the key to successful

* MacGregor Instrument Co., Needham, Mass.

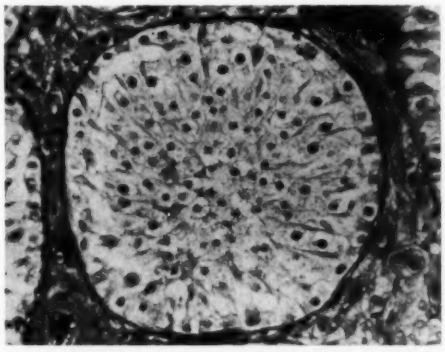

Fig. 2—Single seminiferous tubule from a field of tissue obtained with a 12-gauge needle. H & E stain; original magnification, x 150.

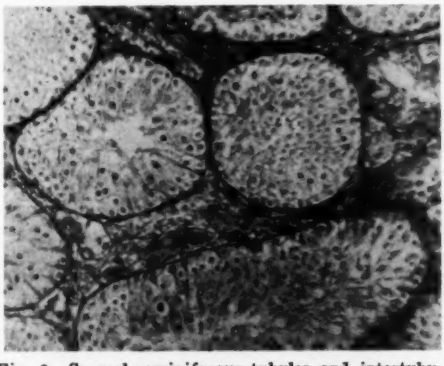

Fig. 3—Several seminiferous tubules and intertubular tissue obtained with a 12-gauge needle. H & E stain; original magnification, x 75.

recovery of a tissue plug from the needle. Fixation should be continued for approximately four hours in Bouin's solution. The cutting edges of the trocar and split needle must be very smooth and sharp or the fragile parenchyma will be distorted. Sharpening on a leather strap is an excellent way to prepare the instruments. Sharpness of the cutting edges should be confirmed by examination under low-power lens magnification.

The skin was not sutured at the site of incision and after-care consisted of the systemic administration of an antibiotic for several days.

To date, 50 biopsies of normal bulls and several biopsies of abnormal bulls have been made with the 12-gauge needle with only one failure to obtain an adequate sample for histologic study. One case of orchitis followed biopsy in a bull that had not been given antibiotics following biopsy. This experience emphasizes the need for prophylactic administration of antibiotics in addition to sterile operative procedure. The biopsy samples have contained an average of 45 seminiferous tubules in cross section with the intertubular tissue intact. Figure 2 is a photomicrograph of a typical single tubule, and figure 3 is a photomicrograph of a typical field of tissue taken by this technique.

A subsequent report [7] relates the short-term and long-term effects of biopsies on the testicular cytology and spermatogenesis in the bull.

SUMMARY

This is the report of a successful technique for testicular biopsy in the bull which is a slight modification of Barker's technique. A 12-gauge Vim-Silverman split needle was used. Testicular samples averaging 45 tubules with little distortion in cross section were obtained.

A ⅛-inch incision made through the skin and tunics over the dorsolateral quadrant of the testicle provided the means for inserting the trocar-cannula toward the center of the testicle. A longer split needle then replaced the cannula and cut a plug of parenchyma which could be removed intact for fixation and histologic study. No significant hemorrhage followed and no suturing of the incision was required. Of 50 biopsy samples obtained by this method, only one was unsuitable for histologic examination and only 1 animal developed orchitis after biopsy.

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SUMMARIO IN INTERLINGUA

Le Agulia de Vim-Silverman Calibre 12 pro Biopsia Testicular del Tauro

Es reportate un successose methodo pro le biopsia testicular in le tauro. Un findite agulia de Vim-Silverman, calibre 12, esseva usate. Le specimens testicular obtenite habeva al media 45 tubulos, e le section transversal monstrava un basse grado de distortion.

Un incision de ⅛ pollice per le pelle e le tunicas supra le quadrante dorsolateral del testiculo provideva le porta de entrata del trocar-cannula. Illo esseva inserite visante al centro del testiculo. Un plus longe findite agulia reimplaciava alora le cannula e secava un tappo de parenchyma que poteva esser extrahite intacte pro fixation e studios histolog'c. Nulle significative hemorrhagia sequeva, e nulle suturation del incision esseva requirite. In un serie de 50 specimens bioptic obtenite per iste technica, solmente un esseva inusabile pro le examine histologic, e solmente un del animales disveloppava orchitis post le intervention.

Changes in Blood Constituents in Sheep Following Prolonged Daily Administration of Iodine^{131}

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THIS REPORT represents a summary of the cellular and biochemical changes occurring in the blood of sheep given 0.005 to 1,800.0 μc. of I^{131} daily for one to six years.

MATERIALS AND METHODS

The experimental design and management of the purebred Suffolk sheep utilized in the study were described in a previous report.[5] The levels of I^{131} fed and the duration of feeding for the original ewes are shown (table 1). The period of study of the offspring actually expresses age, since offspring were exposed to radioiodine for their entire life, including *in utero* existence and the suckling period. At weaning, they were placed on the same I^{131} feeding regimen as their dams and blood samples were not taken until this time. No offspring from ewes given 1,800 μc. per day were available for study, since none survived beyond five days. In one group, 5-month-old lambs from control ewes were given 240 μc. daily.

TABLE 1—Duration of Sampling Period for Experimental Sheep

	Months of exposure to		
μc. I^{131}/Day	Original* lambs	Original** ewes	Offspring†
1,800.0	....	14	....
240.0	12	15	12
135.0	....	8	9
45.0	....	12	9
15.0	....	15	18
5.0	....	49	74–99
1.5	....	42	36
0.5	....	42	36
0.15	....	32	72
0.005	....	12	20

* Exposure initiated at 5 months of age. ** Exposure initiated at 2 years of age. † Does not include *in utero* exposure.

Blood was taken by jugular puncture using a 20-gauge needle and evacuated tubes. Heparin was used as an anticoagulant for cellular studies. Methods for the determination of cellular and chemical constituents have been reported previously.[11] Hemoglobin was determined as alkaline hematin in the earlier studies and by the cyanmethemoglobin method[6] in later studies.

RESULTS

Seasonal trends and variations due to age differences were previously reported[11]; therefore, results are compared on the basis of control versus treated animals. Since complete tabulation of all results would produce a ponderous report, only constituents that significantly vary* from the control values at some period in the study are given.[4]

At 1,800 μc.—No changes were found in values for hemoglobin, erythrocytes, nonprotein nitrogen (NPN), inorganic phosphorus, and calcium. After seven to eight months of I^{131} feeding, ewes had cholesterol values that were lower than those of the controls. Creatinine levels were elevated, especially at three to four months after the initiation of I^{131} feeding. A downward trend in leukocyte and lymphocyte counts occurred, the depression being significant by the second month (table 2). Serum protein-bound iodine (PBI) analyses were not available for this group.

At 240 μc.—A depression in the lymphocyte counts of the ewes was highly significant following four months of I^{131} feeding. Leukocyte values were not as consistently lowered as were the lymphocyte values, and they returned to normal (control) levels after cessation of I^{131} feeding (table 3).

Creatinine values in the original ewes were elevated after five to six months of I^{131} feeding (table 3). The PBI determinations were not started until after the end of the experimental period, but the values were found to be low (0.85 ± 0.21 μg./100 ml.) at that time. No significant differences between control and treated ewes were found for hemoglobin, erythrocyte, NPN, protein,

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* $P < 0.02$ (the probability that the difference is due to chance is less than 2 per cent).

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TABLE 2—Blood Values for Ewes Fed 1,800 μc. of I^{131} per Day

Constituent	Exposure (months)	Treated		Control	
		No.*	Mean	No.*	Mean
Leukocytes (thousands/cmm.)	1	5	10.34 ± 0.75**	49	10.36 ± 0.31**
	2	9	8.40 ± 0.59	45	11.40 ± 0.41
	3	6	7.34 ± 0.36	47	11.35 ± 0.38
	4	4	7.42 ± 1.07	45	10.46 ± 0.33
	5	4	6.35 ± 0.36	44	9.55 ± 0.31
	6	4	6.21 ± 0.46	33	9.57 ± 0.28
	7–8	4	7.51 ± 1.10	47	9.40 ± 0.31
	9–11	8	4.56 ± 0.35	69	8.87 ± 0.29
Lymphocytes (thousands/cmm.)	1	5	5.90 ± 0.49	49	6.24 ± 0.28
	2	9	5.13 ± 0.39	45	6.88 ± 0.31
	3	6	4.03 ± 0.28	47	7.55 ± 0.31
	4	4	3.05 ± 0.68	45	6.80 ± 0.28
	5	4	2.62 ± 0.32	44	6.19 ± 0.30
	6	4	2.50 ± 0.44	33	6.48 ± 0.22
	7–8	4	2.55 ± 0.24	47	6.24 ± 0.24
	9–11	8	2.05 ± 0.14	69	5.59 ± 0.20
Cholesterol (mg./100 ml.)	1–2	14	67.2 ± 4.6	40	71.2 ± 2.5
	3–4	11	70.4 ± 7.6	37	74.2 ± 2.9
	5–6	9	51.3 ± 4.6	37	58.0 ± 2.2
	7–8	4	39.7 ± 1.4	19	60.0 ± 2.7
	9–11	8	44.3 ± 2.5	10	55.8 ± 3.7
Creatinine (mg./100 ml.)	1–2	10	1.81 ± 0.10	40	1.34 ± 0.03
	3–4	9	2.37 ± 0.12	42	1.32 ± 0.02
	5–6	9	1.98 ± 0.09	39	1.25 ± 0.02
	7–8	4	1.75 ± 0.09	18	1.30 ± 0.04
	9–11	5	1.80 ± 0.10	10	1.35 ± 0.06

* Total number of tests. ** Standard error of the mean.

TABLE 3—Blood Values for Ewes Fed 240 μc. of I^{131} per Day

Constituent	I^{131}	Experimental period (months)	Treated		Control	
			No.*	Mean	No.*	Mean
Leukocytes (thousands/cmm.)	Exposed	0	42	9.85 ± 0.32**	84	10.28 ± 0.25**
		1 2	48	9.32 ± 0.33	85	10.40 ± 0.24
		3–4	49	10.96 ± 0.41	91	11.37 ± 0.28
		5–6	48	8.33 ± 0.31	98	10.02 ± 0.23
		7–8	35	8.40 ± 0.29	57	9.68 ± 0.25
		9–10	25	8.02 ± 0.55	52	8.88 ± 0.31
		11–12	35	7.67 ± 0.56	40	8.91 ± 0.40
		13–14	38	7.54 ± 0.44	21	8.88 ± 0.36
	Unexposed	1–2	19	6.88 ± 0.31	29	9.40 ± 0.37
		3–4	8	8.95 ± 0.53	36	10.06 ± 0.32
		5–6	13	7.68 ± 0.66	33	9.46 ± 0.35
		7–19	37	7.67 ± 0.25	194	8.37 ± 0.14
		20–32	15	8.92 ± 0.18	115	8.35 ± 0.19
Lymphocytes (thousands/cmm.)	Exposed	0	44	6.43 ± 0.19	84	6.74 ± 0.18
		1–2	48	5.96 ± 0.21	85	6.50 ± 0.19
		3–4	48	6.64 ± 0.26	92	7.23 ± 0.22
		5–6	46	4.92 ± 0.21	89	6.50 ± 0.21
		7–8	35	4.81 ± 0.17	57	6.56 ± 0.19
		9–10	26	3.81 ± 0.18	52	5.69 ± 0.22
		11–12	34	4.02 ± 0.22	40	5.58 ± 0.26
		13–14	38	3.79 ± 0.16	18	5.74 ± 0.39
Creatinine (mg./100 ml.)	Exposed	0	11	1.20 ± 0.06	20	1.27 ± 0.04
		1–2	22	1.34 ± 0.05	41	1.34 ± 0.04
		3–4	18	1.31 ± 0.05	31	1.27 ± 0.03
		5–6	24	1.40 ± 0.04	50	1.28 ± 0.02
		7–8	19	1.56 ± 0.09	24	1.30 ± 0.04
		9–10	9	1.79 ± 0.17	19	1.29 ± 0.04
	Unexposed	5–6	12	1.88 ± 0.10	7	1.33 ± 0.06

* Total number of tests. ** Standard error of the mean.

TABLE 4—Blood Values for Offspring of Ewes Fed 240 μc. of I^{131} per Day

Constituent	Age (months)	Treated		Control	
		No.*	Mean	No.*	Mean
Leukocytes (thousands/cmm.)	5–6	11	8.58 ± 0.82**	16	11.41 ± 0.58**
	7–8	28	9.09 ± 0.38	30	10.71 ± 0.42
	9–10	19	8.15 ± 0.51	31	11.05 ± 0.54
	11–12	16	5.62 ± 0.28	18	10.27 ± 0.43
Lymphocytes (thousands/cmm.)	5–6	11	5.93 ± 0.82	16	8.03 ± 0.51
	7–8	28	5.07 ± 0.31	30	7.50 ± 0.47
	9–10	19	3.58 ± 0.29	30	8.14 ± 0.48
	11–12	16	3.18 ± 0.24	18	7.25 ± 0.44
Erythrocytes (millions/cmm.)	5–6	13	12.88 ± 0.73	17	14.02 ± 0.61
	7–8	28	10.91 ± 0.33	30	11.96 ± 0.20
	9–10	20	9.96 ± 0.49	32	11.27 ± 0.11
	11–12	16	9.47 ± 0.36	17	11.63 ± 0.28
Creatinine (mg./100 ml.)	5–6	15	1.77 ± 0.12	15	1.25 ± 0.04
	7–9	12	2.08 ± 0.14	17	1.21 ± 0.06
NPN (mg./100 ml.)	5–6	14	44.4 ± 1.8	15	37.6 ± 2.8
	7–9	12	45.0 ± 4.1	17	34.8 ± 1.9
Glucose (mg./100 ml.)	5–6	15	41.8 ± 2.6	15	64.6 ± 4.4
	7–9	12	45.5 ± 6.0	17	55.2 ± 1.0
Calcium (mg./100 ml.)	5–6	15	10.61 ± 0.39	17	12.02 ± 0.19
	7–9	12	12.65 ± 0.65	17	13.63 ± 0.24
Phosphorus (mg./100 ml.)	5–6	14	8.81 ± 0.58	15	6.21 ± 0.28
	7–9	9	7.44 ± 1.06	18	6.59 ± 0.28

* Total number of tests. ** Standard error of the mean.

alkaline phosphatase, cholesterol, glucose, calcium, or phosphorus values.

In comparison to controls, offspring of the ewes given 240 μc. of I^{131} per day were found to have lower leukocyte and lymphocyte values during the initial sampling period. Creatinine was elevated and glucose lowered during this period. Calcium and inorganic phosphorus values had an altered relationship at five to six months, calcium being lower and phosphorus higher than in the controls (table 4).

At the age of 7 to 9 months, treated lambs had higher NPN values than did controls; their erythrocyte levels were depressed from this time until the experimental period was terminated because of the reduction in numbers of animals (table 4). Hemoglobin and cholesterol values were only slightly below those of control lambs ($P<0.05$). Other tests, used for the ewes, produced no significant results. The PBI analyses were not available.

After six months of treatment, control ewes' offspring, first given I^{131} (240 μc. I^{131} per day) at 5 months of age, had lower lymphocyte values than did control lambs. The PBI values were found to be depressed and creatinine values were elevated after two months of I^{131} treatment. No significant differences were observed in phosphorus values, but serum calcium values were lower during the last test period (table 5). After nine months of treatment, lambs had hemoglobin, cholesterol, and NPN levels similar to those of control animals. Erythrocyte and glucose values were not determined.

At 135 μc.—The PBI levels of ewes fed 135 μc. of I^{131} per day were significantly lower than the controls after three to four months of exposure. Creatinine had increased and leukocytes decreased after seven to eight months exposure (table 6). Creatinine and PBI values did not return to control levels after cessation of I^{131} feeding. No changes were observed in leukocyte values for these offspring by the time they were 9 months old.

At 45 μc.—A decrease in PBI and an increase in creatinine values were noticeable in ewes after a 12-month feeding period (table 7), but leukocyte values did not change. The PBI and creatinine values had not reached control levels 18 months after cessation of I^{131} feeding. Values for the lambs from these ewes were comparable to control lambs at 9 months of age.

At 15 μc.—The PBI values for these ewes were significantly lower than those of the control sheep 19 to 22 months after I^{131} feeding was initiated (table 8), while

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TABLE 5—Blood Values for Control Ewes' Offspring Fed 240 μc. of I^{131} per Day

Constituent	Exposure (months)	Treated		Control	
		No.*	Mean	No.*	Mean
Lymphocytes (thousands/cmm.)	1–2	8	7.75 ± 0.75**	12	6.43 ± 0.55**
	3–4	12	6.80 ± 0.56	9	6.19 ± 0.25
	5–6	9	5.37 ± 0.67	8	6.82 ± 0.91
	7–9	19	5.26 ± 1.58	18	7.20 ± 0.41
PBI (μg./100 ml.)	1–2	12	2.69 ± 0.26	9	2.50 ± 0.25
	3–4	9	1.18 ± 0.29	12	2.80 ± 0.18
	5–6	12	0.91 ± 0.19	12	3.37 ± 0.26
	7–8	9	0.68 ± 0.16	6	2.53 ± 0.32
Creatinine (mg./100 ml.)	1–2	12	1.30 ± 0.09	12	1.29 ± 0.04
	3–4	12	1.45 ± 0.08	12	1.20 ± 0.02
	5–6	12	1.72 ± 0.05	12	1.30 ± 0.10
	7–8	12	2.07 ± 0.07	12	1.23 ± 0.05
Calcium (mg./100 ml.)	1–2	6	11.92 ± 0.31	10	11.81 ± 0.19
	3–4	12	12.37 ± 0.15	12	12.17 ± 0.22
	5–6	11	12.29 ± 0.21	12	12.57 ± 0.14
	7–8	12	10.95 ± 0.17	8	12.26 ± 0.28

* Total number of tests. ** Standard error of the mean.

leukocyte and creatinine values were still considered to be the same as control values. No significant results were found in the offspring of these ewes one year after weaning.

At 5 μc.—A decrease in leukocyte values occurred in the original ewes during the 4th year of I^{131} feeding (table 9). No changes were apparent in either PBI or creatinine levels.

First-year offspring of these ewes showed lower PBI values during the entire experimental period, especially after the second year. Creatinine and leukocyte levels were not consistently changed. Lower leukocyte values were obtained during the third, fifth, and sixth year of I^{131} exposure, while creatinine was elevated during the fifth year and early in the seventh year (table 10) of life.

Creatinine and PBI values in the second-year offspring of these ewes did not differ from control offspring until the fifth year of I^{131} feeding (table 11). From the few results gathered from second generation offspring, no change was seen.

At 0.005, 0.15, 0.5 and 1.5 μc.—No significant differences were observed between

TABLE 6—Protein-Bound Iodine, Creatinine, and Leukocyte Values for Ewes Fed 135 μc. of I^{131} per Day

Constituent	I^{131}	Experimental period (months)	Treated		Control	
			No.*	Mean	No.*	Mean
PBI (μg./100 ml.)	Exposed	1–2	22	4.52 ± 0.32**	5	5.24 ± 0.56**
		3–4	6	1.94 ± 0.66	5	4.91 ± 0.18
		5–6	5	1.71 ± 0.39		
		7–8	15	0.88 ± 0.20	3	3.67 ± 0.64
	Unexposed	1–2	3	0.49 ± 0.15		
		18–20	40	0.85 ± 0.38	19	3.54 ± 0.14
Creatinine (mg./100 ml.)	Exposed	1–2	9	1.41 ± 0.05	6	1.40 ± 0.06
		3–4	7	1.39 ± 0.05	6	1.25 ± 0.16
		5–6	4	1.42 ± 0.13		
		7–8	14	1.47 ± 0.06	5	1.08 ± 0.07
	Unexposed	1–2	8	1.50 ± 0.07		
		18–20	40	1.65 ± 0.18	19	1.27 ± 0.04
Leukocytes (thousands/cmm.)	Exposed	1–2	24	10.23 ± 0.36	21	11.36 ± 0.52
		3–4	12	8.17 ± 0.61	21	9.08 ± 0.38
		5–6	9	7.97 ± 0.50	16	8.05 ± 0.38
		7–8	18	7.99 ± 0.43	19	9.46 ± 0.41
	Unexposed	1–2	15	7.22 ± 0.48	28	8.65 ± 0.41
		18–20	36	9.37 ± 0.31	20	9.92 ± 0.58

* Total number of tests. ** Standard error of the mean.

TABLE 7—Blood Values for Ewes Fed 45 μc. of I^{131} per Day

Constituent	I^{131}	Experimental period (months)	Treated No.*	Treated Mean	Control No.*	Control Mean
PBI (μg./100 ml.)	Exposed	1–4	25	4.00 ± 0.31**	10	5.08 ± 0.28**
		5–8	20	2.40 ± 0.24	3	3.67 ± 0.47
		9–12	21	2.51 ± 0.18	7	3.30 ± 0.37
	Unexposed	1–2	6	1.19 ± 0.19	8	2.66 ± 0.19
		16–18	40	1.11 ± 0.06	19	3.54 ± 0.14
Creatinine (mg./100 ml.)	Exposed	1–4	29	1.28 ± 0.04	12	1.32 ± 0.03
		5–8	19	1.32 ± 0.08	5	1.08 ± 0.02
		9–12	22	1.26 ± 0.06	5	1.00 ± 0.04
	Unexposed	1–2	6	1.52 ± 0.05	8	1.20 ± 0.11
		16–18	40	1.49 ± 0.04	19	1.27 ± 0.04

* Total number of tests. ** Standard error of the mean.

the values for control sheep and sheep or their offspring fed 0.005 to 1.5 μc. of I^{131} per day over the feeding periods shown in table 1.

DISCUSSION

Methods frequently used for differentiating hypothyroid, hyperthyroid, and euthyroid states involve the estimation of PBI and cholesterol values in serum or plasma.[7-10 16, 17] A decrease in PBI was found [5] to follow decreased thyroid avidity for I^{131}, but a depression rather than an elevation in serum cholesterol was detected in the ewes given 1,800 μc. Mullick [14] found that thyroidectomized sheep have a lower neutral fat and cholesterol fractions than do normal controls.

TABLE 8—Protein-Bound Iodine Values for Ewes Fed 15 μc. of I^{131} per Day

Exposure (months)	Treated No.*	Treated Mean	Control No.*	Control Mean
1–6	31	3.94 ± 0.25**	10	5.08 ± 0.28**
7–12	29	3.12 ± 0.24	10	3.41 ± 0.28
13–18	23	3.00 ± 0.16	23	3.16 ± 0.21
19–22	19	1.86 ± 0.19	5	3.62 ± 0.43

The PBI values are expressed as micrograms per 100 ml.
* Total number of tests. ** Standard error of the mean.

Protein-bound iodine values were not obtained early in the experimental period for ewes fed 1800 μc. and 240 μc. or for the lambs of ewes fed 240 μc.; however, control lambs fed 240 μc. of I^{131} per day were found to have lowered PBI levels after three to four months feeding. Decreased I^{131} uptake by the thyroid gland was readily evident after six months in ewes fed 135 and 45 μc.,[5] while PBI values were lowered after four and 12 months, respectively.

Greater differences in physiologic effects and thyroid I^{131} avidity in the offspring than in their dams may be explained on the basis of exposure to I^{131} during the fetal and neonatal periods.[3] No change in PBI was apparent in the ewes given 5 μc., but their first-year offspring showed lower values, especially after two years of I^{131} feeding. No explanation can be given for the late changes in PBI values of the second-year offspring. It was also noticed that they had higher thyroid I^{131} uptake.[5]

TABLE 9—Leukocyte Values for Ewes Fed 5 μc. of I^{131} per Day

Exposure (months)	Treated No.*	Treated Mean	Control No.*	Control Mean
0–12	245	10.30 ± 0.16**	430	10.31 ± 0.11**
13–24	70	8.75 ± 0.22	86	8.83 ± 0.24
25–36	169	7.69 ± 0.16	199	8.16 ± 0.17
37–48	14	6.37 ± 0.37	115	8.37 ± 0.19
49	19	6.00 ± 0.22	14	8.27 ± 0.44

Leukocyte values are expressed as thousands per cubic millimeter.
* Total number of tests. ** Standard error of the mean.

Increased creatinine values appeared to follow the depression of thyroid activity in all animals except ewes given 15 μc. and 5 μc.; however, the ewes given 15 μc. were examined for only 22 months. Other studies have reported increased protein catabolism following thyroidectomy or thiouracil treatment in rats.[1, 12, 15] Elevated NPN values were obtained only in the offspring of the ewes given 240 μc. These lambs had clinical signs of hypothyroidism during the first 2 months of life.[5] They also had lowered glucose, calcium, and erythrocyte values and increased phosphorus levels. Loss in the efficiency of intestinal absorption [2] in the young lambs may partially explain

TABLE 10—Blood Values for First-Year Offspring of Ewes Fed 5 μc. of I^{131} per Day

Constituent	Age (months)	Treated		Control	
		No.*	Mean	No.*	Mean
Leukocytes (thousands/cmm.)	5–12	41	11.02 ± 0.49**	43	10.83 ± 0.36**
	13–24	31	10.36 ± 0.40	49	10.75 ± 0.32
	25–36	128	7.97 ± 0.16	115	9.12 ± 0.16
	37–48	10	7.69 ± 0.55	74	8.45 ± 0.17
	51–56	115	7.71 ± 0.15	92	9.19 ± 0.21
	60–62	52	4.77 ± 0.11	52	5.73 ± 0.17
	73–74	12	6.07 ± 0.20	16	7.99 ± 0.62
PBI (μg./100 ml.)	13–24	14	3.63 ± 0.29	10	5.08 ± 0.28
	25–36	60	2.26 ± 0.12	15	3.44 ± 0.24
	37–48	44	1.94 ± 0.13	15	3.41 ± 0.20
	51–56	115	2.34 ± 0.08	91	4.26 ± 0.08
	60–62	52	2.34 ± 0.10	52	3.46 ± 0.10
	73–74	10	2.33 ± 0.18	16	4.62 ± 0.18
Creatinine (mg./100 ml.)	5–12	25	1.30 ± 0.17	29	1.24 ± 0.04
	13–24	14	1.27 ± 0.05	16	1.27 ± 0.03
	25–36	55	1.19 ± 0.02	15	1.10 ± 0.04
	37–48	45	1.53 ± 0.04	14	1.42 ± 0.08
	51–56	110	1.23 ± 0.02	87	1.12 ± 0.02
	60–62	42	1.38 ± 0.05	45	1.31 ± 0.04
	73–74	11	1.55 ± 0.10	16	1.01 ± 0.08

* Total number of tests. ** Standard error of the mean.

these changes, or they may be a reflection of debilitated physical condition. The control lambs fed 240 μc. of I^{131} per day, but not exposed to I^{131} during fetal development, had decreased calcium values only after eight months of feeding. No generalized pathologic changes in the kidneys or parathyroid glands were found in any of the animals.[5]

Leukocyte depression in the ewes and lambs followed alteration of thyroid activity as measured by I^{131} uptake. However, the leukocyte values of ewes fed 135 and 240 μc. of I^{131} per day were within a normal range following cessation of I^{131} exposure, in spite of continued severe hypothyroidism. Sheep given 5 μc. of I^{131} per day had decreasing leukocyte values when thyroid damage was only moderate.[5] These results suggest that radiation damage to hematopoietic tissue may account for the leukocyte depression. A transient reduction in the leukocyte count after therapeutic doses of I^{131} has been reported in man.[13]

SUMMARY

The prolonged administration of 1,800, 240, 135, 45, 15, and 5 μc. of iodine^{131} per day was found to produce hematologic values differing from control levels. In adult ewes, a decrease in PBI values was the first change in a blood constituent due to I^{131} feeding at all levels except 5 μc. This effect was followed by an increase in creatinine values in all sheep except those

TABLE 11—Blood Values for Second-Year Offspring of Ewes Fed 5 μc. of I^{131} per Day

Constituent	Age (months)	Treated		Control	
		No.*	Mean	No.*	Mean
PBI (μg./100 ml.)	5–12	15	4.76 ± 0.35**	11	3.58 ± 0.24**
	13–24	44	4.07 ± 0.15	39	4.28 ± 0.17
	25–36	26	3.37 ± 0.16	20	3.94 ± 0.24
	39–43	85	3.91 ± 0.14	63	4.36 ± 0.14
	48–50	44	3.32 ± 0.47	33	3.95 ± 0.36
	63–64	24	1.45 ± 0.16	30	3.88 ± 0.15
	73–74	24	1.94 ± 0.12	30	3.39 ± 0.14
	95–99	9	1.51 ± 0.15	28	2.84 ± 0.13
Creatinine (mg./100 ml.)	5–12	15	1.33 ± 0.02	14	1.25 ± 0.05
	13–24	47	1.28 ± 0.02	39	1.18 ± 0.02
	25–36	21	1.44 ± 0.02	18	1.38 ± 0.07
	39–43	88	1.29 ± 0.01	63	1.28 ± 0.04
	48–50	48	1.31 ± 0.02	31	1.44 ± 0.02
	63–64	24	1.58 ± 0.09	28	1.23 ± 0.02
	73–74	24	1.37 ± 0.05	30	1.14 ± 0.01
	95–99	9	1.62 ± 0.09	29	1.21 ± 0.03

* Total number of tests. ** Standard error of the mean.

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fed 15 and 5 μc. per day. In sheep maintained on I^{131} for an extended period, depression in the leukocyte count was observed at the 1,800, 240, 135, and 5 μc. per day level; however, in sheep given 5 μc., the depression was slight and did not appear until four years after I^{131} feeding was initiated. Generally, the time of appearance of changes in blood constituents was shortened with increasing dose levels and by initiating exposure at an earlier age. Additional changes, observed in animals exposed to I^{131} *in utero* and during early life, included decreased erythrocyte counts, decreased glucose and calcium levels, and increased NPN and phosphorus values at the 240 μc. per day level (the highest feeding level resulting in significant numbers of viable offspring).

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SUMMARIO IN INTERLINGUA

Alterationes del Constituentes del Sanguine in Oves post le Prolongate Administration Diurne de Iodo^{131}

Esseva trovate que le prolongate administration diurne de doses de 1.800, 240, 135, 45, 15, e 5 μc de iodo^{131} resultava, in oves, in valores hematologic differente ab le valores de controlo. In oves adulte, reduceite nivellos de iodo ligate a proteina esseva le prime effecto hematologic del alimentation de I^{131} in omne le doses studiate excepte illo de 5 μc. Iste effecto esseva sequite per un augmento del valores de creatinina in omne le oves excepte illos recipiente 15 o 5 μc de iodo^{131} per die. In oves recipiente I^{131} durante prolongate periodos de tempore, un depression del numeration leucocytic esseva notate con dosages diurne de 1.800, 240, 135, e 5 μc. Tamen, in le oves recipiente 5 μc, le depression esseva leve e comenciava manifestar se solmente quatro annos post le initiation del alimentation de I^{131}. A generalmente parlar, le declaration del alterationes in le constituentes del sanguine esseva accelerate con augmentos del dosage e initiationes del exposition a etates plus juvene. Alterationes additional, observate in animales exponite a I^{131} in utero e durante le prime phases del vita, includeva reduceite numerationes erythrocytic, reduceite nivellos de glucosa e calcium, e elevate nivellos de nitrogeno non-proteinic e de phosphoro con un dosage de 240 μc per die (que esseva le dosage maximal compatibile con numeros significative de prole viabile).

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Granular Structures in Brains of Apparently Normal Dogs

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DURING histopathologic studies in toxicity tests of a group of dogs, several unidentified, spherical, eosinophilic granular structures were found in the brain and spinal cord. They were observed most frequently in serial sections of the medulla and, less frequently, in the pons and anterior cervical cord of dogs 4 to 5 years of age or older. They were seldom found in dogs less than 1 year of age. These structures had not been recognized previously, and reports describing them could not be found in the accessible veterinary literature. However, similar structures, considered to be degenerating axis cylinders, have been reported to occur in man,[1,2] principally in older people. Those in man frequently are found in association with diffuse demyelinating disease, but apparently are not related to any specific neurologic or pathologic entity. Also, the structures in man were reported as occurring in the white matter in various sites of the brain and apparently were not located principally in the medulla, as reported here for the dog.

In toxicologic studies of new compounds, unknown or previously unidentified lesions must be characterized in order to be properly evaluated. Therefore, a series of detailed studies was made to determine the nature of the granular structures. The purpose of this report is to describe their histologic characteristics in the dog and to give the results of certain histochemical procedures which were carried out in an effort to identify them and evaluate their significance.

MATERIALS AND METHODS

In a preliminary survey of 30 dogs, estimated to be 4 years of age or more, 12 (40%) were found to contain one or more of the granular structures in the medulla. Only when the structures occurred in the medulla was it possible to demonstrate them in other sites.

In order to determine whether the structures might be fixation artifacts, 15 dogs, estimated to be 5 years of age or more, were selected for critical study. These were allotted to three groups of 5 each, with each group having at least 2 dogs of either sex. None of the dogs showed any clinical abnormalities. The dogs in the first group were killed by electrocution, and were immediately exsanguinated. Representative specimens of brain and spinal cord were then fixed in 10 per cent formalin. Dogs in the second group were exsanguinated immediately after being placed under deep pentobarbital anesthesia, and tissues were fixed in 10 per cent formalin. The dogs in the third group were anesthetized with sodium pentobarbital and perfused with a small amount of sodium chloride followed by a 10 per cent formol-saline solution. Representative tissues then were preserved in 10 per cent formalin. Tissues from all the dogs were placed in fixative immediately after death.

A general preliminary histologic study was made of the brain and spinal cord of each dog. Selected tissues were then sectioned serially and stained by appropriate methods. For routine study, tissue sections were stained with hematoxylin and eosin and azure-eosin. For general differential studies, phosphotungstic-acid-hematoxylin, Gomori's trichrome, and van Gieson's stains were employed. For demonstrating special structures, the following methods were used: Perl's Prussian blue test for iron; the periodic acid-Schiff reaction for polysaccharides, mucopolysaccharides, glycoproteins, and glycolipoids; the Alcian blue reaction for acid mucopolysaccharide; Gram's tissue stain for fibrin; the Luxol fast blue-cresyl fast violet for myelin and certain intracellular structures; the Spoerri silver stain for neurofibrils; the Ziehl-Neelsen acid-fast stain; Ralph's method for hemoglobin; and Feulgen's test for desoxyribonucleic acid.[2-6,9]

RESULTS

The granular structures were found in the medullae of 13 of the 15 dogs and there were no significant differences in any of the three groups. Thus, the different methods of euthanasia and fixation, either with or without prior perfusion, did not appear to increase or reduce the number or characteristics of forms found in the various dogs. Ten of the dogs with granular structures in the medulla also had them in the anterior cervical cord, and 3 dogs with medullary structures had them also in the pons.

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The authors appreciate the assistance of Dr. Karl Mayer, Ruhamah Hannah, and M. E. Weidlich in the preparation of this report.

The structures found in the medulla were located most often in Goll's tract and nucleus (fig. 1) and less frequently in other areas of the medulla. They also were found with some regularity in the posterior pons and in the fasciculus gracilis of the anterior cervical cord. In no case was there any appreciable alteration of surrounding neural elements. Neuronal degeneration was not evident nor was there any cellular reaction in the contiguous tissues. Frequently, normal neurons were found in close proximity to a granular structure. These results show that the granular structures are not artifacts.

Nature of the Granular Structures.—When stained by ordinary histologic methods, the structures differed appreciably in size and morphology (fig. 2–5). The smallest forms measured approximately 5 μ in diameter and usually contained rather large oval granules. The entire mass appeared to be circumscribed by a poorly delineated membrane, believed to be a remnant of the myelin sheath. Structures stained vividly with hematoxylin and eosin and Gomori's trichrome were found to be only weakly positive or, in many cases, negative to the Alcian blue and periodic acid-Schiff. The larger structures, which occasionally measured up to 35 μ in diameter, were poorly circumscribed and composed of aggregates of both fine and coarse granules of various sizes. These also stained vividly with hematoxylin and eosin and trichrome stains, but were negative to the periodic acid-Schiff and Alcian blue. With the Spoerri silver stain, all of the granular structures reacted only slightly less vividly than did the meshwork of nerve fibers in the area and, in a few instances, the intensity was greater. With Luxol blue, the structures had a particulate interior with poorly defined granules. Phosphotungstic-acid-hematoxylin demonstrated well-defined, yellowish brown particulate masses. The forms failed to react with Gram's, Perl's Prussian blue, Ralph's, and acid-fast stains and with Feulgen's test.

The results of these various procedures suggest that the structures are products of degeneration, involving principally the axis cylinders. The small forms appear to be swollen axis cylinder balls which have tended to retract from the surrounding fibers. There is some evidence that a remnant of myelin sheath tends to circumscribe them. The larger, fine-granular forms apparently represent fragmentation of the smaller isolated axis cylinder balls and appear to be a terminal stage in degeneration. The morphologic appearance of the very large forms suggests that some of them might also represent degenerated neurons. However, this point was not adequately proved.

DISCUSSION

Although specific neurologic tests were not made of the dogs used in the experiment, general clinical observations failed to show any neurologic disturbance, and

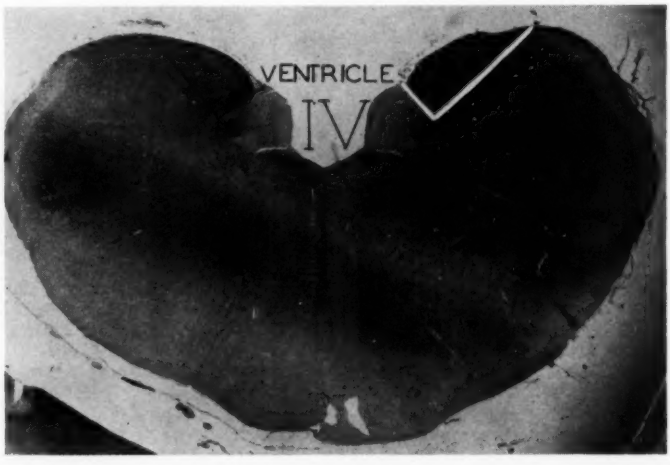

Fig. 1—Transverse section of canine medulla immediately anterior to closure of fourth ventricle. The structures (degenerating axis cylinders) are found most often in the gracilis tract and nucleus as shown. H & E stain.

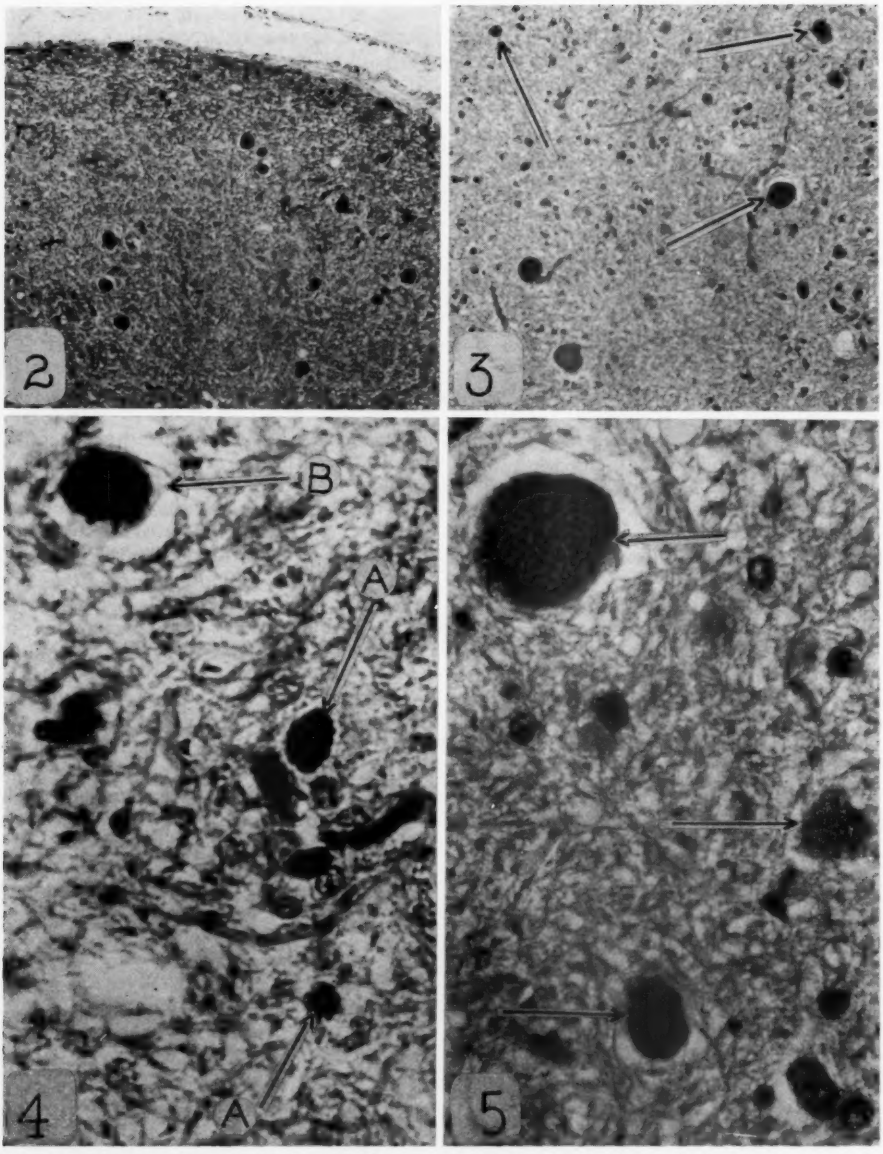

Fig. 2—Low-power view of area shown in figure 1. Approximately a dozen dark structures can be seen in the field. Gomori's trichrome stain; x 35.

Fig. 3—Slightly higher magnification of areas shown in figures 1 and 2. Notice variation in size of the structures (arrows). H & E stain; x 65.

Fig. 4—High-power view of same areas shown in figures 1 and 2. Notice the ovoid, swollen granules (A) and the apparent remnant of a membrane (B). Gomori's trichrome stain; x 320.

Fig. 5—High magnification of structures shown in figures 3 and 4. Notice the normal appearance of contiguous tissues and the differences in granularity of the structures (arrows). H & E stain; x 420.

dogs were considered to be normal clinically. Histologically, there was no evidence of neuronal damage which would normally produce clinical signs. Evidently, therefore, the degenerating axis cylinders are without tangible neurologic or pathologic significance. This finding compares closely with the situation in man, in which no significance has been attached to the presence of similar structures.

A consideration of the genesis of the structures involves a host of various possibilities, some of which are not associated with diseases in any species other than man. However, several of them might be considered in this instance, since the granular structures appear to be degenerating axis cylinders and are identical in the two species. Pellagra and sprue of man are accompanied by marked neuronal degeneration as well as by systemic signs and lesions.[8,10] Wernicke's sign, which is considered to result from thiamine deficiency and occurs in both man and lower animals, is also characterized by neuronal degeneration, together with marked proliferation of astroglia, microglia, and capillaries. A variety of changes, primarily those associated with encephalomalacia, are found in carbon monoxide poisoning.[1,11] None of these alterations holds any similarity to the degenerating axis cylinder forms.

Neither do the structures appear to be consistent with any of the changes observed in presenile or senile cerebral disease in man,[7] such as the senile plaque, Alzheimer basket, or the granulovacuolar change of Simchowicz. Furthermore, there was no evidence of viral, bacterial, fungal, or protozoal infections in any of the dogs, nor any evidence of demyelinization from any cause.

It is conceivable that anoxia could produce alterations such as these. Despite the wide range of conditions under which anoxia of brain tissue might occur, such as disturbances in oxidative metabolism, changes in blood supply, and permeability of the blood-brain barrier, no evidence suggestive of any of these conditions was seen. Considering the wide variation in background of these dogs, such as breed, environment, sex, and age, it is unlikely that any genetic error of metabolism could be involved. Also, there was no clinical or pathologic evidence of chronic heavy metal intoxication. It is conceivable that a more thorough understanding of the circulation in the affected areas might elucidate a possible cause.

The absence of clinical signs and significant histologic changes in other areas of the central nervous system, together with an apparent increase in the number of forms with age, suggest that the axis cylinder degeneration develops insidiously, and probably is a nonspecific defect of aging. On the basis of current evidence, it does not appear to be associated with any specific disease or neurologic change.

SUMMARY

In a preliminary survey of 30 dogs that were at least 4 years of age, spherical, eosinophilic, granular structures were found in the medullae of 12 (40%). In previous examinations, these structures were seldom found in dogs less than 1 year of age. A critical histologic and histochemical study of the structures found in 13 of 15 clinically normal dogs, at least 5 years old, indicated the structures were degenerating axis cylinders having no neurologic or pathologic significance. No explanation of the genesis can be given. It is suggested that, since the number of degenerating axis cylinders increases with age, they probably are a nonspecific defect of aging.

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SUMMARIO IN INTERLINGUA

Structuras Granular in le Cerebros de Apparentemente Normal Canes

In un investigation preliminar de 30 canes de al minus 4 annos de etate, 12 (40%) se distingueva per le presentia de spherie e eosinophilic structuras granular in le medulla. In previe examines, tal structuras esseva trovate rarmente in canes de minus que 1 anno de etate. Un studio critic—histologic e histochimic—de iste structuras trovate in 13 ex 15 clinicamente normal canes de al minus 5 annos de etate resultava in le conclusion que il se tracta hic de axones in stato degeneratori sin signification neurologic o pathologic. Nulle explication del genese de iste structuras pote esser presentate. Viste que le numero del axones degenerante cresce con le etate del individuo, le suggestion es formulate que illos es probabilemente un defecto non-specific del vetulation.

A Clinical and Pathologic Study of 130 Neoplasms of the Mouth and Pharynx in the Dog

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THIS SURVEY was undertaken to correlate the clinical and pathologic features of oral and pharyngeal neoplasms in the dog. Many of the publications in this field are individual case reports or simple listings of tumor types seen in the mouth and pharynx. The histopathologic features of oral neoplasms have been described in considerable detail, but information concerning clinical aspects, gross pathology, and biologic behavior of these neoplasms has often been lacking. Most veterinary textbooks contain little information on canine oral cancer. It is hoped that this study will help to fill this gap in our knowledge of oral tumors.

MATERIALS AND METHODS

One hundred and ten cases of oral and pharyngeal neoplasms were diagnosed in a total of 34,215 dogs examined at the University of Pennsylvania Veterinary Clinic from July 1, 1952, until October 1, 1958, inclusive. Of these dogs, 73 were referred and 37 were brought directly to our clinic. An additional 20 tumors were obtained from biopsy specimens submitted by veterinarians in the Philadelphia area. Neoplasms arising from bone, major salivary glands, or the cutaneous surface of the lips (unless they invaded the oral mucosa) were excluded from this series. Diagnosis was made by histopathologic examination in all but one case (an odontoma). All tissue sections were fixed in 10 per cent formalin and routinely stained with hematoxylin and eosin. In a few cases, Giemsa stains were used to differentiate mast cell tumors from other round cell neoplasms.

MALIGNANT MELANOMA

The 43 melanomas (table 1, fig. 1–7) arose from the gingiva (27 dogs), labial mucosa (6 dogs), palate (5 dogs), and buccal mucosa (5 dogs). In 2 dogs (6 and 12), the tumor involved both the upper and lower surfaces of the opposing gingivae. This involvement may represent spread by contact of malignant tissue against normal tissue or else it is the result of multicentric tumor origin. Males were affected six times as often as females. The average age incidence was 10.4 years and 37 (88%) of the dogs were 7 years of age or older. One third of the tumors occurred in Cocker Spaniels (most of which were black), and the majority of the remaining ones were in animals with heavily pigmented oral mucosae.

In the 23 dogs that were necropsied, 74 per cent had metastases to regional lymph nodes and 65 per cent had lung involvement. Metastases were also observed in such distant sites as the brain, meninges, pituitary body, striated muscle, pleura, pericardium, heart, prostate, pancreas, adrenal glands, ileum, omentum, and eyes. Six of the 8 dogs with either buccal or labial melanomas had metastases to the tonsils. Five of these had metastases to the lungs and the sixth had involvement of the bronchial lymph nodes.

Many melanomas were solidly pigmented, some were partially pigmented, and a few were completely amelanotic. The latter type, which tends to occur in young dogs, presents a diagnostic problem both to the clinician and the pathologist.

The gross pathology of oral melanomas can best be described by placing them into several groups according to the presence or absence of gross pigmentation in either the primary or metastatic lesions: group I, the primary tumor is pigmented and metastatic tumors are pigmented; group II, the primary tumor is pigmented and metastatic tumors are pigmented and nonpigmented; group III, the primary tumor is pigmented and metastatic tumors are nonpigmented; group IV, the primary tumor is nonpigmented and metastatic tumors are pigmented; and group V, the primary tumor is nonpigmented and metastatic tumors are nonpigmented.

Most of the tumors in this study fall into group I, with groups II and III being represented by two cases each and groups IV and V by one case each.

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TABLE 1—Summary of 43 Melanomas of the Mouth and Pharynx

Dog No.	Age (yr.)	Breed	Sex	Clinical signs	Location	Regional lymph node affected	Distant metastases	Method of diagnosis	Remarks
1	10	Cocker Spaniel (color unknown)	M	Rapid growth of tumor, fetor oris, bleeding from mouth for 6 mo.	Gum (canine area).	Not known.	Not known.	B	.....
2	12	Irish Setter	M	Spongy mass, bleeding from mouth.	Gum.	Not known.	Not known.	B	.....
3	10	German Shepherd Dog Chowchow crossbred	M	Swelling of jaw, dysphagia, unable to open mouth.	Lower L. gum (molar area).	Not known.	Not known.	B	Tumor invaded temporomandib. joint.
4	8	Collie	M	.....	Gum.	Not known.	Not known.	B	.....
5	13	Cocker Spaniel (black and white)	M	Rapid growth, bleeding from mouth for 5 wk.	Upper L. gum.	R. and L. mandib. hard and large.	Probable.	B	Tumor infiltrated anterior hard palate.
6	17	German Shepherd Dog crossbred	M	Fetor oris.	Upper R. gum (3–4 premolar); lower R. gum (3–4 premolar).	Not known.	Not known.	B	Upper gum lesion, 2.5 x 2.0 x 1.5 cm.; opposing lesion, 1.5 x 1.5 x 1.0 cm.
7	....	Poodle	....	.....	Gum.	Not known.	Not known.	B	.....
8	3	Not known	M	.....	Gum.	Not known.	Not known.	B	.....
9	13	Scottish Terrier	M	Swelling of face and bleeding from mouth for 6 mo. after extraction of incisor tooth.	Upper gum.	R. and L. mandib. prescap.	Adrenals (bilateral).	N	Tumor infiltrated hard and soft palate.
10	10	Mixed breeding	M	Maxillary swelling for 1 wk.	Upper R. gum (premolar area).	R. mandib.	Ant. mediastinum.	B & N	Marked local bone destruction, tumor infiltrated hard palate.
11	14	Cocker Spaniel crossbred	M	Signs appeared rapidly 3 wk. after tooth extraction.	Lower L. gum (premolar and molar area).	L. mandib.	Absent.	N	Marked destruction of adjacent mandible.
12	11	Cocker Spaniel (black)	M	Bleeding, fetor oris, emaciation over 1-yr. period.	Lower L. gum (premolar area); upper L. (premolar).	Negative.	Lungs, pleura, omentum, heart, liver, pancreas, meninges, pituitary gland.	B & N	Primary tumor invaded mandible, post. pharynx, and floor of the mouth.

TABLE 1 (continued)—Summary of 43 Melanomas of the Mouth and Pharynx

Dog No.	Age (yr.)	Breed	Sex	Clinical signs	Location	Regional lymph node affected	Distant metastases	Method of diagnosis	Remarks
13*	8	Cocker Spaniel (red)	M	Tumor apparent for 1 mo.	Lower R. gum (premolar area).	Negative.	Absent.	B & N	Had the L. eye enuculated because of malignant melanoma 7 mo. before gum tumor seen. Gum tumor invaded turbinate area. Also had medullary pheochromocytoma of L. adrenal gland.
14	7	Bedlington Terrier	M	Fetor oris for 1 wk.	Lower L. gum (premolar area).	Negative clinically.	Not known.	B	19 mo. after tumor was removed, osteomyelitis of affected jaw was curetted; biopsy was negative for melanoma. Pathologic fracture developed 1 mo. later and euthanasia was performed.
15	7	Doberman Pinscher	M	Dysphagia, fetor oris, and hypersalivation for 3 wk.	Lower ant. gum (medial aspect).	Negative.	Probable.	B	Recurred 22 mo. later; dog was very thin and losing weight rapidly.
16	12	Cocker Spaniel (black)	M	Fetor oris, bloody saliva for 6 mo. after tooth extracted.	Lower ant. gum (incisor area).	Not known.	Not known.	B	7 mo. after tumor was removed, euthanasia was carried out because of recurrent mass.
17	14	Cocker Spaniel (black)	M	Gum swelling for 1 yr., pain, dysphagia.	Upper R. gum.	Negative.	Not known.	B	No follow-up.
18	14	Cocker Spaniel (black)	M		Lower ant. gum.	R. and L. mandib.	Lungs.	N	Also had a ceruminal adenocarcinoma of the external ear.
19	8	English Setter	M	Gum swelling.	Lower R. gum (canine area).	R. mandib. enlarged.	Absent.	B	.....
20	14	Mixed breeding	F	Gum swelling for 4 mo.	Upper post. gum.	Negative.	Lungs, pericardium, ant. mediastinum.	B & N	.....

TABLE 1 (continued)—Summary of 43 Melanomas of the Mouth and Pharynx

Dog No.	Age (yr.)	Breed	Sex	Clinical signs	Location	Regional lymph node affected	Distant metastases	Method of diagnosis	Remarks
21	2	Basset Hound	M	Gum swelling for 2 wk.	Lower L. gum (premolar and molar area).	Negative.	Absent.	B & N	Radiotherapy resulted in 4-mo. regression of tumor and necrosis of mandible, followed by very rapid recurrence.
22	13	Poodle	F	Gum swelling for 2 wk.	Lower R. gum.	L. mandib. enlarged.	Not known.	B	.....
23	8	Long-Haired Dachshund	F	Gum swelling for 1 yr.	Upper L. gum.	Not known.	Not known.	B	.....
24	9	Terrier	M	Proptosis of L. eye for 1 wk.	Upper R. gum (molar area).	R. and L. mandib.	Absent.	B	Tumor invaded maxillary sinus and hard palate.
25	8	Miniature Poodle	F	Gum swelling noticed for 2 days.	Upper R. gum.	R. mandib.	Lungs.	B & N	Some lung lesions pigmented, others amelanotic.
26	2	Boxer	F†	Proptosis of R. eye for 10 wk.	Upper R. gum (molar area).	R. mandib.	Absent.	N	Wide invasion of palate, R. turbinates, and R. maxillary sinus.
27	8	Cocker Spaniel (red)	F	Gum swelling noticed for 2 wk.	Upper gum (incisor area).	Negative clinically.	Not known.	B	Lesion somewhat pedunculated and amelanotic.
28	9	Kerry Blue Terrier	M	Choking spells for 3 wk. and a rapidly enlarging mass.	Junction of hard and soft palate.	R. and L. mandib.; R. and L. retrophar.	Lungs, bronchial lymph node.	N	.....
29	9	Dachshund	M	Dysphagia for 3 wk.	Soft palate.	R. and L. mandib.; R. and L. retrophar.	Lungs.	N	.....
30	8	Scottish Terrier	M	Dyspnea, epistaxis for 1 mo.	Hard palate.	R. mandib.	Lungs.	N	Tumor infiltrated turbinate area and maxillary sinuses; also had an acinar adenoma of the pancreas.

TABLE 1 (continued)—Summary of 43 Melanomas of the Mouth and Pharynx

XUM

TABLE 1 (continued)—Summary of 43 Melanomas of the Mouth and Pharynx

Dog No.	Age (yr.)	Breed	Sex	Clinical signs	Location	Regional lymph node affected	Distant metastases	Method of diagnosis	Remarks
31**	9	English Setter	M	None.	Soft palate.	R. and L. mandib., prescap.	Bronchial lymph nodes, lungs, diaphragm, pleura, omentum, mesentery, pituitary, pericardium, kidneys.	N	Also had a feminizing Sertoli cell tumor of a scrotal testis with iliac node metastases, a hemangioma of the spleen, leiomyoma of the stomach, and a bile duct adenoma of the liver.
32	13	Cocker Spaniel (black)	M	Dyspnea for 1 mo., unilateral nasal discharge for 1 wk.	Hard and soft palate.	R. and L. mandib.	Bronchial lymph nodes.	N	.....
33	7	Dalmatian crossbred	M	Rapid growth for 6 wk. Recurred in 7 mo. and spread to both tonsils and ipsilateral mandibular node. All involved tissues excised.	Mucocutan. commissure of R. lip.	R. and L. mandib., R. and L. retrophar., R. and L. tonsils.	Lungs, ant. mediastinal and bronchial nodes, protate, meninges.	B & N	Recurrent lip lesion, both tonsils, and mandib. node excised. At necropsy 6 mo. later, recurrent tonsillar melanomas were present and all regional lymph nodes were involved.
34	13	Cocker Spaniel (black)	M	Rapidly growing swelling.	Mucocutan. commissure of lower L. lip.	L. mandib.	Not known.	B	Recurred 6 wk. after first operation and 3 wk. after the second.
35	8	Cocker Spaniel (black)	M	Tumor present for 4–5 yr. Doubled in size last year.	Upper R. labial mucosa.	R. mandib. enlarged	Negative radiograph of thorax.	B	A huge amelanotic mass widely infiltrating the labial mucosa and surrounding soft tissues.
36	13	Cocker Spaniel (black)	M	Fetor oris for 3 mo.	Lower R. labial mucosa.	R. tonsil.	Lungs.	N	.....
37	4	German Shepherd Dog	M	Lesion noticed for 2 wk.	Mucocutan. commissure of L. lip.	R. and L. mandib., L. tonsil.	L. thyroid gland, bronchial lymph nodes.	B & N	Lesion recurred after incomplete removal. This recurrent tumor was removed and and a second recurrence was observed 14 mo. later. Died 3 wk. after recurrence.

TABLE 1 (continued)—Summary of 43 Melanomas of the Mouth and Pharynx

Dog No.	Age (yr.)	Breed	Sex	Clinical signs	Location	Regional lymph node affected	Distant metastases	Method of diagnosis	Remarks
38	12	Mixed breeding	M	Swelling of lip noticed for 2–3 mo.	Mucocutan. commissure of R. lip.	R. mandib.	Probably had metastases when euthanasia performed.	B	Rapid downhill course after surgery. Severe dyspnea, loss of weight. Euthanasia 3 mo. later.
39	12	Mixed (black)	M	Very rapid growth of lesion for 3 wk.	R. buccal mucosa.	R. mandib., R.retrophar., R. tonsil.	Lungs, R. eye.	N	A huge necrotic black mass greatly distending the buccal cavity.
40	10	Cocker Spaniel (black)	M	Lesion noticed for 6 mo. Buccal tumor removed and recurred 3 mo. later. At this time tonsillar metastasis was observed.	L. buccal mucosa.	L. tonsil.	Lungs, liver.	B & N	Buccal lesion was pigmented but tonsillar metastasis was amelanotic and initially diagnosed as carcinoma. Buccal tumor recurred 2 mo. after the second operation and euthanasia was performed.
41	6	Cocker Spaniel (black)	M	Pigmented swelling, which recurred 6 mo. after excision, was again excised.	L. buccal mucosa.	Negative.	Lungs, pleura, pericardium, heart, mediastinum.	B & N	No local recurrence after second excision, although visceral lesions were present at necropsy 9 mo. later.
42	12	Airedale Terrier	M	No signs noticed by owner.	L. buccal mucosa.	L. mandib., L. prescap., L. tonsil.	Lungs, kidneys, mediastinum, intercostal muscles.	B & N	Two attempts at removal were followed by rapid recurrence.
43	11	Labrador Retriever	M	2-wk. rapid growth.	L. buccal mucosa.	L. mandib., L. retrophar., L. prescap.	Lungs, kidneys, mediast., intercostal muscle, pulmonary artery, R. frontal lobe of brain.	B & N	.....

B = biopsy. N = necropsy.
* *See* ref. 11. ** *See* ref. 1. † spayed.

Fig. 1—Heavily pigmented pedunculated malignant melanoma (arrow) arising from the lower gingiva near the left canine tooth (C). The tongue (T) has been pulled to one side (dog 15, table 1).

Fig. 2—Nonpigmented malignant melanoma arising from the lateral surface of the lower gingiva (dog 14, table 1).

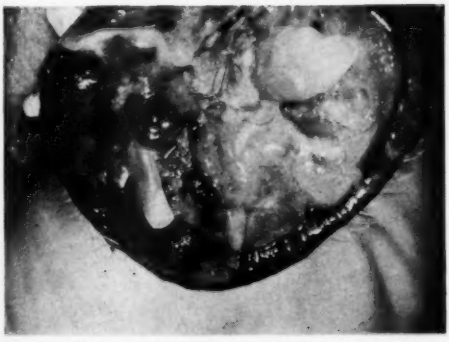

Fig. 3—Malignant melanoma of the anterior lower gingiva (dog 18, table 1). One side of the lesion is heavily pigmented, the other is amelanotic. Notice displacement of the incisor and canine teeth by the tumor.

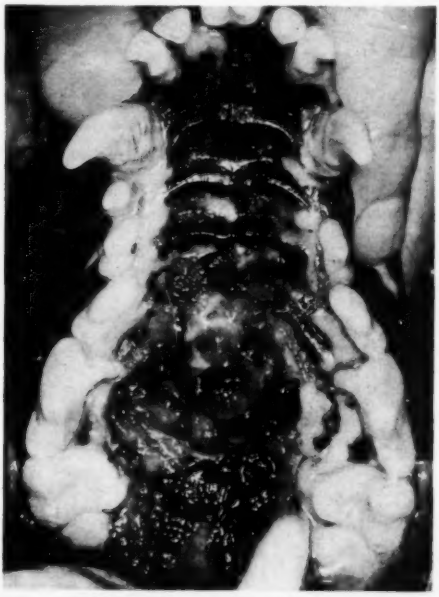

Fig. 4—Malignant melanoma involving the posterior two thirds of the hard palate (dog 30, table 1). This ulcerated pigmented tumor had invaded the turbinate area and both maxillary sinuses.

All of the melanomas appeared to be malignant microscopically. The most important single finding was the presence of varying amounts of melanin granules in the cytoplasm of the tumor cells. In a few instances, the abundance of pigment obscured cytologic detail, but the presence of tissue infiltration, tumor-cell emboli in vessels, and metastatic deposits in regional lymph nodes confirmed the diagnosis of malignancy. In most cases, the tumor cells appeared to be of ectodermal origin, but, in a few instances, a mixed epithelial-mesenchymal cell pattern or a neural type of pattern was evident. Nuclear pleomorphism and abundant mitoses were present in almost every case.

Melanomas were usually rapidly growing lesions which often infiltrate adjacent soft tissues and bone. The clinical course was variable, ranging from one week to five years, but euthanasia was often indicated several months after the tumor was first seen. In some cases, the metastatic lung nodules were so small that they could not be detected radiographically. Involved lymph nodes were often normal in size and

XUM

TABLE 2—Summary of 25 Squamous Cell Carcinomas of the Mouth and Pharynx

Dog No.	Age (yr.)	Breed	Sex	Location	Clinical signs	Metastases	Method of diagnosis	Remarks
1	12	Boston Terrier	M	Gum (premolar area).	Rapid growth in alveolus 2 mo. after premolar extracted. Dyspnea.	Negative clinically.	B	Bone destruction so extensive that the nose could easily be bent laterally. Infiltration of hard palate and turbinates.
2	12	Cocker Spaniel	....	Upper gum.	.....	Negative clinically.	B	.....
3	14	Cocker Spaniel	F	Upper gum (4th molar area).	Tumor noticed for 3 years.	Negative clinically.	B	Tumor invaded hard palate and maxillary sinus.
4	9	Boston Terrier	M	L. gum (canine area).	.....	Negative clinically.	B	.....
5	7	Mixed	M	Lower L. gum (between canine tooth and lateral incisor).	Swelling of gum noticed for 1 wk.	Negative clinically.	B	Recurred 3 wk. after initial excision. Second operation included extraction of lateral incisor and curettage. No recurrence 8 mo. later.
6	5	Standard Poodle	F	Lower R. gum (4th molar area).	Gum swelling noticed 1 wk. after tooth extraction; hypersalivation, some bleeding.	Negative radiograph of thorax.	B	X-ray therapy gave temporary regression lasting 3 mo.
7	4	Boxer	F	Lower R. gum (from central incisor to 3rd molar tooth).	Swelling of gum, bleeding from mouth.	Absent.	B & N	Tumor infiltrated around the roots of the affected teeth.
8	10	Boxer	M	Upper R. gum (1st premolar area).	Swelling present 1 mo., recurred 2 wk. after excision.	Negative radiographs of thorax.	B	Tumor invaded labial mucosa. Several courses of x-ray therapy resulted in some regression of growth. Euthanasia performed 6 mo. later due to tumor growth.

TABLE 2 (continued)—Summary of 25 Squamous Cell Carcinomas of the Mouth and Pharynx

XUM

TABLE 2 (continued)—Summary of 25 Squamous Cell Carcinomas of the Mouth and Pharynx

Dog No.	Age (yr.)	Breed	Sex	Location	Clinical signs	Metastases	Method of diagnosis	Remarks
9	8	Collie	F	Upper R. gum (4th premolar area).	Appeared shortly after 4th premolar tooth was extracted.	Negative clinically.	B	.....
10	7	Doberman Pinscher	M	Lower R. gum (lateral incisor and canine area).	Loosening of teeth in area of swelling noticed for 2 wk. Tumor bled readily.	Negative clinically.	B	.....
11	6	Collie crossbred	M	Upper R. gum (central and lateral incisor area).	Loosening of teeth in area of swelling noticed for 2 wk.	Negative radiograph of thorax.	B	Implantation of radon seeds into the tumor resulted in apparent recovery.
12	8	Cocker Spaniel	F	Upper L. gum (canine area).	Rapidly growing friable tumor. Recurred 1 mo. after excision.	Negative radiograph of thorax.	B	.....
13	6	English Setter	F	Lower L. gum (canine and 1st premolar area).	Noticed shortly after extraction of 1st premolar tooth.	Negative radiograph of thorax.	B	X-ray therapy caused tumor to disappear. Four years later, owner thought tumor had recurred and euthanasia was carried out.
14	8	Standard Poodle	F	Lower R. gum (premolar area).	Oral bleeding for 3–4 mo.; loosening of affected teeth.	Negative radiograph of thorax.	B	X-ray therapy resulted in temporary regression of tumor growth.
15	10	Mixed breeding	F	Lower R. gum (canine and incisor area).	Huge mass on gum with loosening of teeth in affected area.	Negative radiograph of thorax.	B	Tumor invaded floor of mouth. Temporary regression of tumor followed roentgen therapy.
16	11	Cocker Spaniel	M	Upper L. gum (canine area).	Noticed for 2 wk.; tumor very large and firm.	Negative radiograph of thorax.	B	.....
17	12	Irish Terrier	M	Upper L. gum (incisor and canine area).	Bleeding from mouth for 1 wk.; spongy mass on gum line.	Negative radiograph of thorax.	B	Tumorous invasion of hard palate. X-ray therapy resulted in regression of tumor for 3 mo., followed by rapid growth and considerable bleeding for next 7 mo. until dog's death.

TABLE 2 (continued)—Summary of 25 Squamous Cell Carcinomas of the Mouth and Pharynx

Dog No.	Age (yr.)	Breed	Sex	Location	Clincial signs	Metastases	Method of diagnosis	Remarks
18	8	German Shepherd Dog crossbred	M	Upper gum.	Swelling and pain in nasal region for 2 mo.	Not known.	B	Nose very flexible due to extensive destruction of nasal bones, turbinates, and hard palate.
19	6	German Shepherd Dog	F	Lower L. gum (incisor area).	Noticed for 4 wk. Excised but soon recurred.	Negative radiograph of thorax.	B	Euthanasia 5 mo. after x-ray therapy because of radiation necrosis of mandible.
20	3	Bassett Hound	F	Upper anterior gum.	Noticed for 6 wk. Rapid growth of tumor.	Negative radiograph of thorax.	B	Tumor undermined ant. 2 ridges of hard palate; x-ray therapy resulted in rapid regression of tumor. 8 mo. later, affected area was reported free of tumor
21	13	Boxer	F	Upper L. gum (2nd premolar area).	Tumor polypoid, 2 cm. in length. Noticed only at necropsy.	Lungs.	N	Also had brain, lung, bronchial lymph node, renal, cardiac, and pancreatic metastases from a recurrent mammary adenocarcinoma.
22	6	German Shepherd Dog	M	Upper L. gum (premolar area).	.....	Not known.	B	.....
23	10	Irish Setter	M	Junction of hard and soft palate on midline.	.....	Absent.	B & N	.....
24	14	Terrier	M	R. pharyngeal mucosa.	Pain, drooling for 1 yr., then unable to open mouth.	Absent.	N	Invasive, firm, greyish tissue invading pharynx, soft palate, and base of tongue, with compression of brain tissue just posterior to pituitary.
25	9	Mixed breeding	M	R. dorsal surface of posterior third of tongue.	Poor appetite and hypersalivation for 2 wk. Ulcerating tongue lesion.	R. mandibular lymph node.	B & N	Local recurrence and regional node metastasis 1 mo. after hemiglossectomy.

B = biopsy. N = necropsy.

consistency, thus misleading the clinician to think that the lesion was still localized.

The prognosis in all oral or pharyngeal melanomas is exceedingly poor [7-9]; we have failed to effect a recovery in a single case.

Fig. 5—Multiple pigmented metastatic foci in the lungs (dog 31, table 1). The primary melanoma arose from the soft palate.

This occurred because of the difficulty encountered in excision of the primary lesion and the early development of metastases to regional lymph nodes and distant sites.

SQUAMOUS CELL CARCINOMA

The 54 carcinomas arose from the tonsils (29 dogs), gingiva (22 dogs), palate (1 dog), pharynx (1 dog), and the tongue (1 dog).

Gingiva.—The average age incidence of gingival carcinomas was 8.4 years, and there was no particular sex or breed incidence (table 2, fig. 8, 9). These carcinomas are usually reddened, friable, and vascular. The history often relates to a tooth extraction with subsequent growth of the tumor. Thus, it is mistakenly thought that the extraction precedes the neoplasm; actually, the carcinoma destroys the periodontal structures, resulting in loosening of the teeth. This cancer has a strong tendency to invade adjacent tissues, particularly bone.

Carcinoma of the gum is a far less malignant lesion than its counterpart in the tonsils. This observation may be related to several possibilities. First, there may be some inherent difference in squamous cell carcinomas arising from the gum as compared to those arising from the tonsils. The second possibility is related to lymphatic drainage. The tonsils are richly supplied with

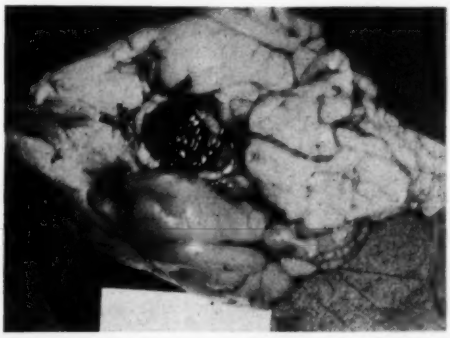

Fig. 6—Same tumor as pictured in figure 5. Pituitary body was replaced by a highly pigmented metastatic melanoma.

efferent lymphatics leading not only to the ipsilateral mandibular or retropharyngeal lymph nodes but also to the contralateral nodes. In 3 of 4 normal dogs in which the right tonsils were injected with Berlin blue dye, there was bilateral staining of the mandibular or retropharyngeal lymph nodes or both. This is probably the reason why tonsillar cancer spreads so readily to regional lymph nodes. The lymphatic drainage of the gums, however, is far more restricted and appears to go only to the ipsilateral mandibular gland. The third possibility is that the tonsils, being primarily lymphoid tissue, offer less resistance to tumor infiltration than does the dense connective tissue of the gingiva and underlying jaw bone.

In 3 of the 22 dogs with gingival carcinomas, there was ipsilateral mandibular

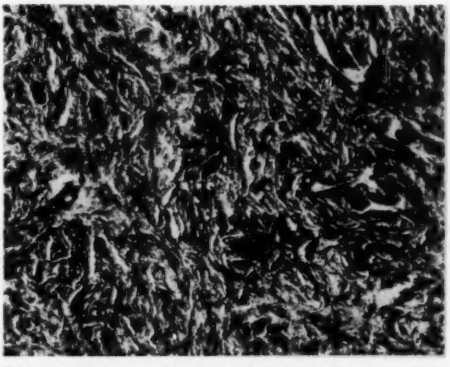

Fig. 7—Same tumor as pictured in figure 1. Notice the masses of melanin pigment scattered throughout the tumor. H & E stain; x 6

XUM

TABLE 3—Summary of 29 Cases of Squamous Cell Carcinoma of the Tonsils

Dog No.	Age (yr.)	Breed	Sex	Duration of signs	Tonsil involved	Mandibular lymph node		Retropharyngeal lymph node		Prescapular lymph node		Other metastases	Method of diagnosis
						Right	Left	Right	Left	Right	Left		
1	6	Not known.	Not known.	1 wk.	L.	..	*	..	..	..	..	.....	B
2	6	Irish Terrier	M	2 wk.	L.	..	*	..	..	..	*	Liver and spleen.	B & N
3	12	Dachshund	M	Coughing for 1 yr.	R.	*	*	..	..	..	..	R. thyroid.	N
4	11	Mixed breeding	M	3 mo.	L.	..	*	..	..	..	..	.....	B
5	2	Mixed breeding	F	2 wk.	R.	*	*	..	..	*	..	R thyroid, lungs.	N
6	9	Dachshund	F	6 wk.	L.	..	*	..	..	..	..	.....	B
7	11	German Shepherd Dog	M	6 wk.	L.	..	*	..	..	..	..	.....	N
8	12	Terrier	F	2 wk.	L.	..	*	..	..	..	..	.....	N
9	10	Cocker Spaniel	F	3 wk.	R.	..	..	*	*	..	..	Lungs.	N
10	16	Wire-haired Fox Terrier	M	6 wk.	R.	*	..	..	..	*	..	Liver, pancreatic lymph node.	N
11	10	Mixed breeding	F	4 mo.	R.	*	..	*	..	..	..	R. thyroid.	N
12	Not known.	Mixed breeding	M	.....	L.	*	*	..	..	..	..	R. thyroid, lungs.	N
13	7	German Shepherd Dog	M	3 mo.	L.	*	*	..	..	..	..	Liver, ribs, lungs.	B & N
14	10	Boston Terrier	M	.....	L.	..	*	..	..	..	..	L. thyroid (clinical).	B
15	5	Mixed breeding	M	5 mo.	R.	* (cl.)	..	..	..	..	..	.....	B
16	9	Hound	M	2 mo.	L.	..	* (cl.)	..	..	..	..	Negative radiograph of thorax.	B
17	6	Boston Terrier	F†	1 yr.	R.	Neg. (cl.)		..	..	..	..	Negative radiograph of thorax.	B
18	11	Mixed breeding	M	.....	R.	*	..	..	..	..	..	.....	B
19	11	Cocker Spaniel	M	6 mo.	L.	..	..	*	..	..	*	Ant. mediastinum, lungs, pericardium, liver, heart, kidneys.	B & N

TABLE 3 (continued)—Summary of 29 Cases of Squamous Cell Carcinoma of the Tonsils

TABLE 3 (continued)—Summary of 29 Cases of Squamous Cell Carcinoma of the Tonsils

Dog No.	Age (yr.)	Breed	Sex	Duration of signs	Tonsil involved	Mandibular lymph node		Retropharyngeal lymph node		Prescapular lymph node		Other metastases	Method of diag-nosis
						Right	Left	Right	Left	Right	Left		
20	2	Airedale Terrier	M	3 wk.	R.	*	..	..	..	..	..	L. and R. thyroid.	B & N
21	5	Springer Spaniel	M	2 mo.	L.	..	*	..	..	..	..	L. thyroid.	B & N
22	11	Cocker Spaniel	M	3 wk.	L.	* (cl.)	* (cl.)	* (cl.)	* (cl.)	..	* (cl.)	Negative radiograph of thorax.	B
23	15	Mixed breeding	M	.....	....		*	Contralateral node.				L. and R. thyroid.	N
24	9	Cocker Spaniel	M	6 wk.	R.	*	..	..	..	..	..	.....	N
25	12	Cocker Spaniel	M	2 mo.	L.	..	..	..	*	..	..	Lungs.	N
26	9	Mixed breeding	M	3 mo.	L.	..	*	*	*	..	..	.....	B
27	14	Mixed breeding	M	1 wk.	R.	*	..	..	..	..	..	Negative radiograph of thorax.	B
28	14	Mixed breeding	M	None.	R.	..	..	*	..	..	..	.....	N
29	11	Mixed breeding	F	4 wk.	L.	*	*	*	*	..	..	Lungs, spleen, liver, diaphragm.	N

B = biopsy. N = necropsy. Cl. = clinically. † Spayed.

lymphadenopathy that was clinically suggestive of metastasis. The other 19 dogs lacked clinical signs of metastasis to regional lymph nodes. Metastasis of the lungs was found in 1 dog (21) which had a small pedunculated gingival tumor.

Gingival carcinoma is locally invasive but slow to metastasize. Because of these characteristics and the marked sensitivity of this tumor to roentgen therapy, carcinoma of the gum appears to have a better prognosis than any other oral malignancy. The more extensive the osteolysis, the poorer the prognosis, as it is more difficult to destroy nests of tumor cells located deep in bone without causing severe radiation necrosis of bone.

TONSILS

Tonsils.—The average age incidence of tonsillar carcinomas was 9.8 years; 20 (71.4%) of the 28 dogs were 9 years of age or older (table 3, fig. 10–14). Males were affected three times as commonly as females and there was no particular breed incidence. The left tonsil was involved in 16 dogs, the right in 12. The chief signs noticed by the owners were dysphagia, 13 dogs; a cervical lump, 11; dyspnea, 9; anorexia, 8; cough, 8; drooling, 8; cervical pain, 6; change in voice, 6; choking, 2; and bleeding from the mouth, 2.

The salient features in the differential diagnosis of tonsillitis, tonsillar carcinoma, and lymphosarcoma are summarized (table 4). Carcinoma of the tonsils in the dog shows a striking parallel to tonsillar cancer in man. The primary lesion is often small and may go unnoticed; whereas, the secondary deposits in the lymph nodes are usually very prominent.[15] Most of the cases that we have seen were previously incorrectly diagnosed as cervical abscess, malignant lymphoma, or salivary gland tumor. In other words, the large metastatic regional lymph node was thought to represent the primary lesion and the mouth and pharynx were never examined. If a careful examination of the tonsils is made, this disease can usually be readily diagnosed. In some dogs, it may be necessary to employ a short-acting intravenous anesthetic to properly examine the tonsillar area. Endotracheal equipment should be readily available in case of airway obstruction. A dog with an upper lateral cervical mass should always be checked for tonsillar carcinoma.

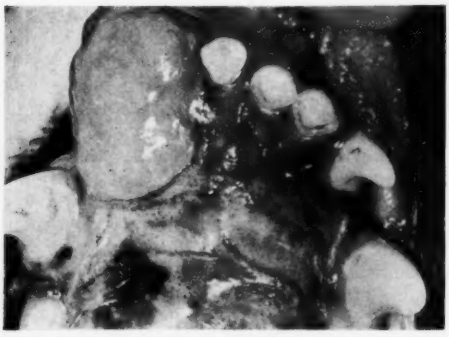

Fig. 8—Squamous cell carcinoma of the gingiva (dog 11, table 2). The tumor grew rapidly following extraction of two incisor teeth.

Fig. 10—Squamous cell carcinoma of the right tonsil (dog 18, table 3). This diffusely ulcerated tumor has widely infiltrated the pharynx and soft palate.

The mandibular lymph nodes were involved in 24 of the 28 dogs with metastasis to lymph nodes. In 23 of the 24 dogs, the ipsilateral node was involved; 8 of these dogs also had metastases to the contralateral lymph nodes. One dog had only contralateral mandibular lymphadenopathy. The retropharyngeal lymph nodes were involved in 8 dogs. In 4 of these, the mandibular lymph nodes were not involved. Spread to ipsilateral retropharyngeal nodes was present in all 8 dogs, 4 of which also had contralateral metastases. Thus, 28 (96%) of 29 dogs had metastases to regional lymph nodes. The ipsilateral prescapular lymph node was involved in 4 dogs. Metastasis to the thyroid gland occurred in 8 dogs. Ipsilateral metastasis to the thyroid gland was present in 5 dogs, contralateral metastasis in 1, and bilateral metastases in 2 others.

Fig. 9—Anaplastic carcinoma of the upper gingiva (dog 16, table 2). Notice extensive invasion of the hard palate, gingiva, and adjacent labial mucosa.

Fig. 11—Squamous cell carcinoma of the right tonsil (dog 13, table 3). This large irregular lesion is displacing the larynx and epiglottis.

XUM

Affected thyroid glands were firm, grayish, nodular, and had varying degrees of enlargement. In many areas, thyroid follicles were compressed or replaced by carcinoma cells. Histologically, tumor emboli were seen to fill myriads of interstitial lymphatics and small veins. Thus, the thyroid gland appears to be an important metastatic pathway for tonsillar carcinoma. In 17 dogs that were necropsied, other sites of metastases were: lungs, 7 dogs; liver, 4; spleen, 2; pericardium and heart, 1; ribs, 1; kidney, 1; anterior mediastinal lymph node, 1; and pancreatic lymph node, 1.

In each of 7 dogs, one of the tonsils was destroyed and replaced by a gray, granular, ulcerating plaque which often involved the adjacent pharynx, soft palate, and tongue. In each of 12 animals, one of the tonsils was slightly enlarged, firm, gray, and nodular, and in each of 10 others a huge cauliflower-like tonsillar mass occluded the pharynx and upper airway.

The regional lymph nodes were usually moderately to massively enlarged; on cut section they were firm, grayish and often contained necrotic centers. Occasionally, necrotic tumor tissue drained through sinus tracts to the skin of the cervical area (dog 21). When a lesion had broken through the capsule of the node, there was often widespread invasion of surrounding blood vessels, striated muscles, and nerves. Dog 24 had a distinct right-side Horner's syndrome (unilateral miosis, enophthalmos, conjunctival hyperemia, and ptosis of the eyelid) due to neoplasic infiltration of the right cervical sympathetic trunk.

Histologically, all of the tumors were typical squamous cell carcinomas which tended to be rather undifferentiated. Widespread tissue invasion and tumor cell emboli in adjacent vessels were commonly observed.

The prognosis of tonsillar carcinoma is extremely poor and none of our dogs recovered. This is due to the infiltrative nature of the primary lesion and the marked propensity to metastasize to regional nodes and distant sites. In the few dogs where treatment appeared feasible, the tonsillar tumor was treated by surgical excision or radiation therapy accompanied by surgical removal of the involved regional nodes.

Dogs 13 [2] and 29 had hypertrophic osteoarthropathy which was undoubtedly related to the pulmonary metastases present in each.

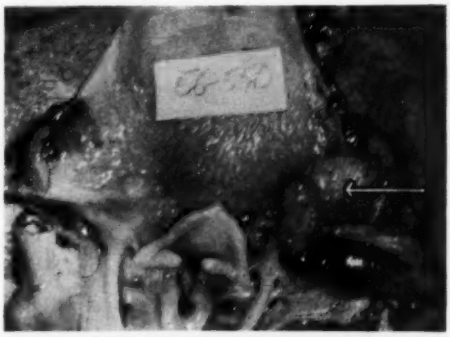

Fig. 12—Squamous cell carcinoma of the right tonsil (arrow). The hard, greyish, nodular lesion is typical of this disease (dog 3, table 3).

Most of the reports of tonsillar carcinoma have come from certain large industrial areas in England.[4-6, 16] Some of these dogs developed bilateral tonsillar tumors; only unilateral neoplasia was noticed in ours. The high incidence of tonsillar neoplasia in England was thought to be related to air pollution by carcinogenic substances. The

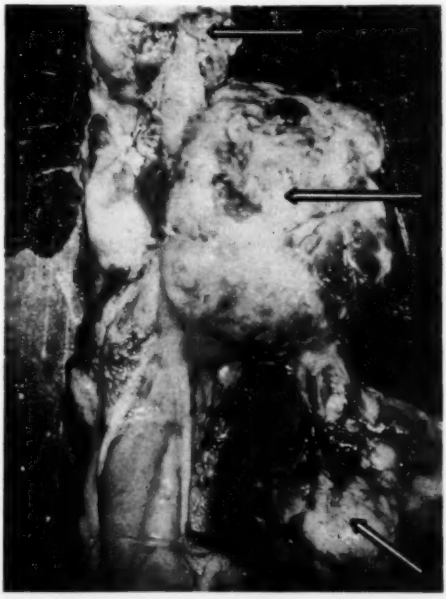

Fig. 13—Squamous cell carcinoma of the right tonsil (dog 10, table 3). The small primary tumor (top arrow) is contrasted to the bulky metastases in the ipsilateral mandibular lymph node (middle arrow) and prescapular lymph node (bottom arrow).

Fig. 14—Squamous cell carcinoma of the tonsil (dog 3, table 3) with normal stratified squamous epithelium (uper left) and diffuse cellular tumor tissue (lower and middle right). H & E stain; x 6.

tonsillar crypts may retain carcinogens for a long time, and this may partially explain why tonsils are so often affected with carcinoma, as compared to the pharyngeal mucosa which is infrequently involved.

There have been very few cases of tonsillar carcinoma [10,12] described in North America; however, this study indicates that this disease is not a rarity, at least in the Philadelphia area. An epidemiologic investigation of this disease on a world-wide basis would undoubtedly reveal much valuable information.

NEUROFIBROSARCOMA

The 18 sarcomas (table 5, fig. 15–19) arose from the gingiva, 10 dogs; soft palate, 2; labial mucosa, 2; buccal mucosa, 2; hard palate, 1; and tongue, 1 dog. The average age incidence was 7.9 years and 9 (53%) of the 17 dogs were 7 years of age or younger. Males were affected twice as often as females, and there was no particular breed incidence.

These tumors often widely infiltrated adjacent soft and bony tissues. Tumors were usually firm, fleshy, and red or pink. Metastasis to regional nodes was absent in

TABLE 4—Differential Diagnosis of Inflammatory and Neoplastic Lesions of the Tonsils

Item	Tonsillitis	Squamous cell carcinoma	Lymphosarcoma
Age incidence	Usually young animals.	Usually old animals (average age 9 years).	Usually middle-aged or old animals (average age lower than for carcinoma).
Size of tonsils	Uniform enlargement—may be 2–3 times normal size. Usually bilateral.	Nodular enlargement; may form huge irregular mass, or tonsil may be replaced by a plaque-like ulcer. Usually unilateral.	Uniform enlargement may become quite large. Usually bilateral.
Color of tonsils	Reddened—may be tiny foci of necrosis or suppuration.	Gray, pinkish-gray, or red.	Pink or red.
Consistency of tonsils	Slightly increased.	Very firm; often lesion is hard.	Much softer than carcinoma.
Surrounding tissues	Normal or hyperemic.	Often diffusely infiltrated with gray, granular, tumor tissue.	Usually normal.
Regional lymph nodes (mandibular and retropharyngeal)	Slight uniform enlargement; may be hot and painful. Usually bilateral.	Moderate to massive enlargement, irregularly lobulated. Node is very firm but not hot or painful. Usually unilateral involvement, but may be bilateral.	Moderate to massive, often irregularly lobulated. Node is not as firm as with carcinoma, no heat or pain. Usually bilateral involvement.
Superficial body lymph nodes	Negative.	May have prescapular adenopathy; rest of nodes negative.	Usually greatly enlarged (also internal nodes, liver, and spleen often involved).
Signs	Fever, gagging, cough, depression, anorexia.	Dysphagia, dyspnea, choking, lateral cervical mass, pain, ptyalism, anorexia, etc.	Very variable; depends on the involvement in body cavities or cervical nodes and not on tonsillar lesions.
Blood findings	Leukopenia or leukocytosis.	Negative.	Leukocytosis with neutrophilia, usually; a few have lymphatic leukemia.
Radiograph of thorax	Negative.	May have metastatic lung nodules.	Often have hilar or anterior mediastinal lymphadenopathy.

XUM

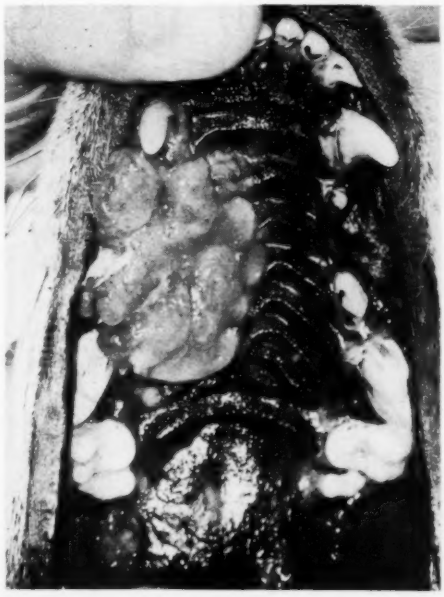

Fig. 15—Neurofibrosarcoma of the upper gingiva, with wide infiltration of the hard palate (dog 5, table 5).

Fig. 16—Neurofibrosarcoma of the soft palate (dog 11, table 5). A piece of Gelfoam sponge is located on the lesion just above the site of a biopsy. Diffuse gingival hypertrophy is also present.

all cases and only the dog with the lingual sarcoma had pulmonary metastases. Thus, the squamous cell carcinomas of the gum and these sarcomas of the gum behaved in a similar manner; *i.e.*, both were highly invasive locally, but had very little tendency to metastasize.

Histologically, these sarcomas were composed of spindle-shaped cells arranged in

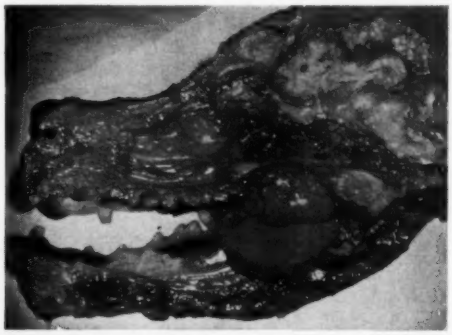

Fig. 17—Same tumor as pictured in figure 16 after sagittal section of the head, showing origin of the tumor from the soft palate and compression of the posterior nares.

Fig. 18—Neurofibrosarcoma of the buccal mucosa (dog 15, table 5). This recurrent neoplasm is diffusely ulcerated and contains much necrotic tissue.

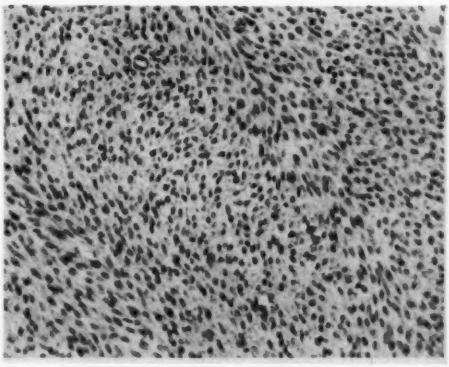

Fig. 19—Neurofibrosarcoma of the tongue (dog 18, table 5), in which the spindle-shaped tumor cells are arranged in interlacing bundles producing a herring-bone pattern. H & E stain; x 6.

XUM

TABLE 5—Summary of 18 Sarcomas of the Mouth and Pharynx

Dog No.	Age (yr.)	Breed	Sex	Location	Clinical signs	Metastases	Diagnosis	Remarks
1	5	English Setter	M	Lower L. gum (canine area).	Rapidly growing necrotic mass; recurred 9 wk. after excision.	Absent.	Neurogenic * sarcoma (N).	Marked osteolysis in tumor area was seen on radiographic examination of the mandible.
2	7	Cocker Spaniel	M	Upper R. gum (canine area).	Sore mouth for 2–3 mo.; teeth in tumor area previously removed.	Absent.	Neurogenic sarcoma (N).	Marked osteolysis in tumor area was seen on radiographic examination of the maxilla. Tumor extended into hard palate and turbinates.
3	9	Terrier	F	Lower L. gum (area of last molar).	Decreased appetite for 2 wk.	Absent.	Neurogenic sarcoma (B & N).	Huge, firm tumor mass which invaded tongue, floor of mouth, pharynx, and buccal mucosa.
4	15	Airedale	M	Anterior lower gum.	Long-standing history of gum tumor.	Absent.	Neurogenic sarcoma (N).	Teeth in tumor area loosened. Surrounding bone relatively unaffected.
5	11	Terrier	M	Upper gum (canine to last premolar).	Pink mucoid discharge from R. nostril for 2 wk.	Negative radiograph of thorax	Neurogenic sarcoma (B).	Tumor invaded hard palate and turbinates. Also had gray nodular mass in left tonsil, resembling squamous cell carcinoma.
6	4	Mixed breeding	F	Anterior upper gum.	Diffuse swelling of muzzle with loosening of teeth for 6–8 wk.	Absent.	Myxosarcoma (B).	Neoplastic invasion of hard palate, anterior turbinates, and muzzle. Severe osteolysis in tumor area was seen on radiographic examination. Lesion was firm and had same color as the normal gum tissue.
7	14	Pekinese	M	Lower L. gum (premolar area).	Found accidentally during teeth cleaning.	Negative radiograph of thorax	Neurogenic sarcoma (B).	.....
8	7	German Shepherd Dog	F	Upper L. gum (incisor to third premolar area).	Gum swelling for 22 mo.; recurred twice after excision.	Negative radiograph of thorax	Neurogenic sarcoma (B).	A low-grade sarcoma was initially diagnosed histologically as an epulis.

TABLE 5 (continued)—Summary of 18 Sarcomas of the Mouth and Pharynx

Dog No.	Age (yr.)	Breed	Sex	Location	Clinical signs	Metastases	Diagnosis	Remarks
9	2	Irish Setter	F	Anterior lower gum.	Rapidly growing tumor noticed for 2 mo.	Negative radiograph of thorax	Neurogenic sarcoma (B).	Recurred twice after surgical removal.
10	2	Cocker Spaniel	M	Upper gum region of last 2 molars.	Enlarged cervical lymph node, firm pink gingival mass invading bone.	Mandibular lymph node on affected side enlarged and and hard.	Neurogenic sarcoma (B).	.....
11	7	Boxer	F	Soft palate.	Bleeding from mouth and dyspnea for 2 days.	Absent.	Neurogenic sarcoma (B).	Lesion very bulky and compressed posterior nares. Invaded posterior portion of hard palate.
12	2	Collie	M	Hard palate.	Bleeding from mouth for 2 wk.	Absent.	Neurogenic sarcoma (B).	Recurred twice after removal. Treatment with thio-TEPA was unsuccessful.
13	1.5	Boxer	M	Soft palate.	Hypersalivation for 1 wk.	Negative radiograph of thorax.	Neurogenic sarcoma (B).	.....
14	13	Scottish Terrier	F	Mucocutaneous junction of anterior lip.	Ulcerative swelling for 3 wk., rapid growth.	Negative radiograph of thorax.	Neurogenic sarcoma (B).	X-ray therapy resulted in disappearance of lesion. No signs of tumor 8 mo. later.
15	10	Doberman Pinscher	M	Labial and buccal mucosa.	Rapidly growing ulcerating mass noticed for 2 mo.	Negative radiograph of thorax.	Neurogenic sarcoma (B).	Recurred 5 wk.after first operation. The second operation resulted in incomplete excision and rapid growth of tumor.
16	8	German Shepherd Dog crossbred	M	L. buccal mucosa.	Large ulcerated mass noticed for 7 mo.	No information.	Neurogenic sarcoma (B).	Tumor incompletely removed; dog not available for follow-up.
17	17	Shetland Sheepdog	M	R. buccal mucosa.	Red, cauliflower-like, friable, 2.5-cm. mass noticed for 3 wk.	No information.	Neurogenic sarcoma (B).	.....
18	5	Standard Poodle	M	R. lateral surface of middle third of tongue.	Fetor oris and bleeding from mouth for 1 wk.	Lungs.	Neurogenic sarcoma (B & N).	Tumor infiltrated into musculature of the tongue.

B = biopsy; N = necropsy.
* Same as neurofibrosarcoma.

TABLE 6—Summary of 15 Miscellaneous Neoplasms of the Mouth and Pharynx

Dog No.	Age (yr.)	Breed	Sex	Location	Clinical signs	Metastasis	Method of diagnosis	Remarks
1	6	Boxer	F	Soft palate.	Detected during physical examination.	No information.	Lymphosarcoma (B).	Presented because of bilateral lymphosarcoma of membrana nictitans.
2	5	Cocker Spaniel	F	R. buccal mucosa.	Rapidly growing swelling of cheek.	R. mandib. lymph node.	Mast cell sarcoma (B).	Multiple submucous nodules which were confluent with metastatic lymph node.
3	6	Boxer	F	Upper gum (1st and 2nd premolar area).	Bleeding from mouth for 1 yr.	No information.	Hemangiosarcoma (B).	Tumor friable, vascular, and necrotic.
4	10	Hound	M	Upper R. gum (molar area).	Rapid growth for 3 mo.	Absent.	Hemangiosarcoma (B and N).	Marked destruction of adjacent maxillary zygomatic, lachrimal, and palatine bones and maxillary sinus.
5	14	Cocker Spaniel	F	Anterior lip.	Soft, red mass in labiogingival area.	Absent.	Trichocarcinoma (B and N).	Initial superficial biopsy revealed squamous cell hyperplasia and lesion, diagnosed as an early squamous cell carcinoma. Tumor widely invaded adjacent gum tissue.
6	8–9	Boxer	M	Lower lip.	Multiple submucosal nodules.	Absent.	Trichocarcinoma (B).	Clinically tumors appeared to arise in the submucosa.
7	1	Dalmatian	M	Upper gum.	Gum swelling, many small teeth present in premolar area.	Absent.	Compound composite odontoma (B).	About 70 teeth in various stages of formation; removed by curettage after reflection of the oral mucosa.

TABLE 6 (continued)—Summary of 15 Miscellaneous Neoplasms of the Mouth and Pharynx

Dog No.	Age (yr.)	Breed	Sex	Location	Clincial signs	Metastasis	Method of diagnosis	Remarks
8	12	Boxer	M	Labial and buccal mucosa (bilateral).	Diffuse reddened lesions.	Regional lymph nodes.	Reticulum cell sarcoma (B).	Clinical course of over 2 yr. before regional node metastases developed.
9	7	English Bulldog	M	R. labial mucosa, tongue.	Rapid weight loss for 1 wk.	R. mandib. lymph node, pericardium, myocardium, overlying 2, 3, and 6 thoracic vertebrae, skin of chest wall.	Lymphosarcoma (N).	A highly unusual case with multicentric origin from various areas in the mouth, pharynx, and epiglottis.
10	5	Wire-Haired Fox Terrier	....	Lower gum.	Swelling of gum for 2 mo.	Absent.	Papilloma (B).	No recurrence after excision.
11	13	Samoyed	M	Upper L. labial mucosa.	2-cm. submucous nodule detected at necropsy.	Absent.	Histiocytoma (N).	Also had perianal adenocarcinoma and diabetes mellitus.
12	6	Cocker Spaniel	M	R. and L. tonsils.	Dysphagia and dyspnea for 2 days.	R. and L. mandibular and retropharyngeal nodes.	Reticulum cell sarcoma (N).	L. tonsil was 3.6 x 2.2 x 1.2 cm.; R. tonsil was 6.3 x 2.2 cm.
13	4	Mixed breeding (Chow)	M	R. and L. tonsils.	Coughing, gagging, and choking for 1 wk.	R. and L. retropharyngeal nodes	Lymphosarcoma (B and N).	L. tonsil was 1.8 x 1.6 x 0.9 cm.; R. tonsil was 1.1 x 0.8 x 0.4 cm. The w.b.c. was 21,900/cmm., with 80% neutrophils and 17% normal lymphocytes.
14	1	Poodle	....	R. and L. tonsils.	No information.	R. and L. retropharyngeal nodes	Lymphosarcoma (B).	2 wk. after surgery, all superficial lymph nodes were enlarged.
15	7	Wire-Haired Fox Terrier	M	L. tonsil.	None.	Absent.	Lymphangioma (B).	2-cm. pedunculated lesion of L. tonsil. No recurrence after tonsillectomy.

B = biopsy; N = necropsy.

Fig. 20—Lymphosarcoma involving both tonsils (dog 13, table 6).

definite interlacing bundles which assumed a herring-bone type of pattern. Some cross sections of these bundles resembled cross sections of nerve fibers with small nuclei of the Schwann cell type. In a few tumors the elongated nuclei tended to line up in parallel fashion. The microscopic similarity of these tumors to normal peripheral nerve sheaths suggests that their origin may be from nerve sheath cells. Cellular anaplasia, increased mitosis, and infiltration of adjacent tissues were commonly observed.

Fig. 21—Lymphosarcomatous nodules in the tongue, pharynx, and epiglottis (dog 9, table 6).

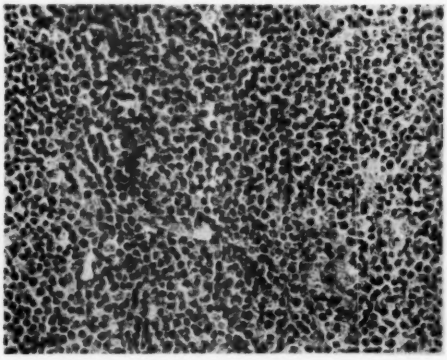

Fig. 22—Same tumor as pictured in figure 20, showing marked cellularity, nuclear pleomorphism, and mitoses. H & E stain; x 6.

The prognosis in most cases is poor due to extensive involvement of the primary tumor. Dog 13, with a lip sarcoma, was successfully treated with radiation therapy. This suggests that radiation treatment may be of some value in cases where the lesion is not too far advanced. Radical surgical therapy alone was not successful in controlling the lesion, and rapid recurrence was the rule.

MALIGNANT LYMPHOMA

The six lymphomas arose from the tonsils and cervical lymph nodes in 3 dogs, membrana nictitans and soft palate in 1, gingival labial and buccal mucosa in 1, and pharynx, tongue, and epiglottis in 1 (table 6, fig. 20–25). Four of these tumors were classified as lymphosarcomas and two as reticulum cell sarcomas. In all of these animals, the tumor arose in the mouth or pharynx, although in dog 14 generalized lymphadenopathy appeared late in the course of the disease. Treatment was unsuccessful in all of these dogs.

MISCELLANEOUS NEOPLASMS

The other malignant neoplasms were hemangiosarcomas of the gingiva (2 dogs), trichocarcinomas of the lip (2 dogs), and mast cell sarcoma of the buccal mucosa (1).

The benign neoplasms observed were histiocytoma of the labial submucosa (1 dog), papilloma of the gingiva (1 dog), lymphangioma of a tonsil (1 dog), and an odontoma in the maxillary area (1 dog). The last lesion was classified as a compound composite odontoma[13] and it produced hundreds of small teeth.

DISCUSSION

Of the 130 neoplasms in this series, 126 (97%) were malignant. The extremely low incidence of benign neoplasms in this series is due mainly to the exclusion of a lesion referred to as epulis, periodontal fibromatous epulis,[8,9] or gingival hypertrophy.[14] This entity is extremely common in brachycephalic dogs, particular the Boxer. We have observed some evidence of this disease in approximately 30 per cent of Boxers over 5 years of age. Typically, this lesion is a firm, fibrous, gingival swelling that is covered by intact oral epithelium. The lesions have the same color as the normal gingival tissues (unlike most neoplasms). These growths are usually sessile, often completely covering the incisor teeth and, to a lesser extent, the premolars or molars. In a few cases, one of the lesions is pedunculated and becomes traumatized during mastication. Multiplicity of involvement is the rule in brachycephalic dogs, but much more localized lesions are usually present in other breeds. In this latter group, faulty alignment of a tooth or excessive tartar deposits usually cause chronic gingival irritation which predisposes dogs to this condition. In the Boxer,[3] a genetic background for the disease may be present. However, since malocclusion is so common in brachycephalic breeds, the resultant chronic irritation to the gums may be of etiologic significance; *i.e.*, dogs with more severe malocclusion may have more severe gum changes (table 7).

On cut section, the affected gum tissue is found to be firm, fibrous, relatively avascular, and may contain osseous foci. Histologically, the lesion is composed of abundant fibrous connective tissue with strands of odontogenic epithelium and foci of lamellar bone formation. In the author's opinion, gingival hypertrophy, as seen in hundreds of affected dogs in our clinic, is a self-limiting disease which lacks the clinical and microscopic criteria of a neoplastic process. It should be considered as a fibroblastic proliferation of gum tissues, probably as a reaction of chronic gingival irritation. For this reason, gingival hypertrophy has not been included with the oral and pharyngeal neoplasms.

A second and far less important reason for the low incidence of benign lesions was the failure to biopsy viral oral papillomas which are seen rather infrequently in this area. In some cases, only a solitary papil-

Fig. 23—Reticulum cell sarcoma of the labial, buccal, and gingival mucosae (dog 8, table 6). The lesions were beet red.

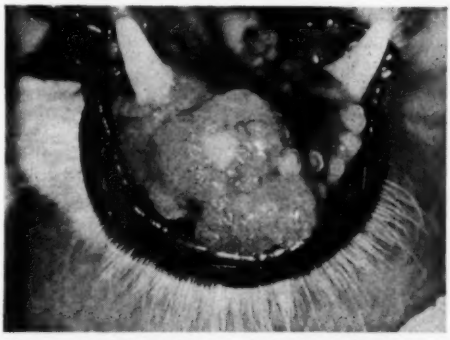

Fig. 24—Trichocarcinoma of the lower lip (dog 5, table 6) with wide invasion of the labial mucosa and the anterior gingiva.

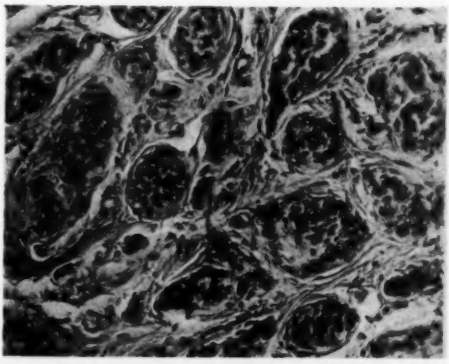

Fig. 25—Same tumor pictured in figure 24, with numerous gland-like structures resembling hair follicles which lie in a fibrous stroma. H & E stain; x 6.

TABLE 7—Summary of 130 Neoplasms of the Mouth and Pharynx in the Dog

Site of origin	Squamous cell carcinoma	Malignant melanoma	Neurogenic* sarcoma	Malignant lymphoma	Hemangio-sarcoma	Mast cell sarcoma	Tricho-carcinoma**	Papilloma	Lymph-angioma	Histio-cytoma	Odontoma	Total
Gingiva	22	28	10	..	2	..	..	1	..	..	..	63
Tonsil	29	....	....	3	..	..	..	..	1	..	..	33
Palate	1	5	3	1	..	..	..	..	..	..	..	10
Labial mucosa**	....	6	2	1	..	..	2	..	..	1	..	12
Buccal mucosa	....	4	2	..	..	1	..	..	..	..	..	7
Tongue	1	....	1	..	..	..	..	..	..	..	..	2
Pharynx	1	....	....	1	..	..	..	..	..	..	..	2
Enamel organ	....	....	....	..	..	..	..	..	..	..	1	1
TOTAL	54	43	18	6	2	1	2	1	1	1	1	130

* One of these tumors was a myxosarcoma. ** Neoplasms of the cutaneous surface of the lips, except those invading the oral mucosa, which were not included in this study.

loma was present. One dog, however, had multiple papillomas covering the labial and buccal mucosae, the gingiva, and the hard and soft palates.

Of the tumors studied, 75 per cent arose from the gingiva and the tonsils. The high frequency of involvement of these two sites may be related to their anatomical structure; *i.e.*, ingested carcinogens may become entrapped between the gums and the teeth or in the tonsillar crypts. Here they could lie for long periods of time and be fairly well protected from the cleansing action of the salivary juices. Inflammatory conditions of the gums and tonsils are commonly observed in dogs. Long-standing chronic irritation or infection of these areas may act as a nonspecific factor which may play a role in malignant transformation in a small percentage of affected animals.

SUMMARY

Of 34,215 dogs examined from July 1, 1952, to October 1, 1958, at the University of Pennsylvania Veterinary Hospital, 130 had oral and pharyngeal neoplasms. Diagnosis was made histopathologically in all but one case (an odontoma).

Malignant melanomas in 43 dogs arose from the gingiva (27 dogs), labial mucosa (6 dogs), palate (5 dogs), and buccal mucosa (5 dogs). Males were affected six times as often as females, and the average age incidence was 10.4 years. The Cocker Spaniel was the breed most commonly affected. Most of the other dogs had heavily pigmented oral mucosa. Of the 23 dogs necropsied, 74 per cent had metastases to regional lymph nodes and 65 per cent had pulmonary involvement. Some had secondary melanomas in such distant sites as the brain, meninges, pituitary body, and eyes. Six of the 8 dogs with either buccal or labial melanomas had tonsillar metastases. The prognosis in oral melanomas is exceedingly poor because of their marked local invasiveness and their propensity to form both lymphatic and hematogenous metastases. Most melanomas are pigmented, but the occasional amelanotic tumor is a diagnostic problem to both the clinician and the pathologist.

Squamous cell carcinomas in 54 dogs arose from the tonsils (29 dogs), gingiva (22 dogs), palate (1 dog), pharynx (1 dog), and tongue (1 dog). The tonsillar carcinomas were highly invasive and metastasized to regional lymph nodes in all but 1

dog. Males were affected three times as often as females, the average age incidence being 9.8 years. There was no particular breed incidence. Of the 17 dogs necropsied, the main sites of distant metastases were lungs (7 dogs), thyroid gland (7 dogs), liver (5 dogs), and spleen (2 dogs). The prognosis in tonsillar carcinoma is extremely poor, as the primary tumor is often inoperable; early metastases almost invariably develop in the ipsilateral and contralateral regional lymph nodes. A significant number of dogs also develop metastases to distant sites. Tonsillar carcinoma rarely has been reported in the United States, although it is a common disease in certain industrialized areas of England where it is thought to be due to air pollution with various carcinogens. This study, made in the Philadelphia area, indicates that tonsillar carcinoma is not as rare as reports would indicate.

Carcinomas of the gingiva behaved quite differently than did those of the tonsils. The average age incidence was 8.4 years and there was no significant sex or breed incidence. Although gingival carcinoma often destroyed adjacent bone, it rarely metastasized. Only 1 dog had pulmonary metastases. The prognosis is better than for any other oral malignancy. In several cases, recovery or prolonged periods of tumor regression were obtained following roentgen therapy or radon implants. The lingual carcinoma widely infiltrated the striated musculature of the tongue and metastasized to the regional lymph node. The palatine carcinoma did not metastasize.

Neurogenic sarcomas in 17 dogs arose from the gingiva (9 dogs), soft palate (2 dogs), labial mucosa (2 dogs), buccal mucosa (2 dogs), hard palate (1 dog), and tongue (1 dog). One myxosarcoma of the gingiva was also seen. The average age incidence was 7.9 years. Males were affected twice as often as females and there was no significant breed incidence. Although these sarcomas were very invasive locally, they had little tendency to metastasize. Only the lingual sarcoma produced secondary lesions in the lungs. These tumors were classified as neurogenic sarcomas because their histologic appearance suggested origin from peripheral nerve sheath cells. The prognosis in most cases was poor due to extensive local involvement of the tumor, although 1 dog was successfully treated by roentgen therapy.

Malignant lymphomas in 6 dogs arose from the tonsils and cervical lymph nodes (3 dogs), membrana nictitans and soft palate (1 dog), gingival, labial and buccal mucosa (1 dog), and pharynx, tongue and epiglottis (1 dog). Four of these growths were classified as lymphosarcomas and two as reticulum cell sarcomas. Treatment was unsuccessful in all 6 dogs.

The other malignant neoplasms were hemangiosarcoma of the gingiva (2 dogs), trichoepithelioma of the lip (2 dogs), and mast cell sarcoma of the buccal mucosa (1 dog).

The benign neoplasms observed were histiocytoma of the labial submucosa (1 dog), papilloma of the gingiva (1 dog), lymphangioma of the tonsil (1 dog), and odontoma in the maxillary area (1 dog). The last lesion was classified as a compound composite odontoma and it produced hundreds of rudimentary teeth.

There were 88 neoplasms in males and 32 in females, a 2.75:1 ratio. The four most common neoplasms of the mouth and pharynx were carcinoma (41.5%), malignant melanoma (33.1%), neurogenic sarcoma (13.8%), and malignant lymphoma (4.6%). The frequency of neoplastic involvement of the various sites was gingiva (48.4%), tonsil (25.4%), labial mucosa (9.2%), hard and soft palate combined (7.8%), buccal mucosa (5.4%), tongue (1.5%), pharynx (1.5%), and enamel organ (0.76%).

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SUMMARIO IN INTERLINGUA

Un Studio Clinic e Pathologic de 130 Neoplasmas del Bucca e del Pharynge in Canes

Inter 34,215 canes examinate inter le 1 de julio 1952 e le 1 de octobre 1958 al Hospital Veterinari del Universitate Pennsylvania, 130 habeva neoplasms oral e pharyngee. Le diagnose esseva facite histopathologicamente in omne le casos, con un exception. In isto il se tractava de un odontoma.

Le serie includeva 88 neoplasmas in masculos e 32 in femininas. Isto es un proportion de 2,75 a 1. Le quatro neoplasmas le plus commun in bucca e pharynge esseva carcinoma (41,5%), melanoma maligne (33,1%), sarcoma neurogene (13,8%), e lymphoma maligne (4,6%). Le frequentia del affection neoplastic del varie sitos esseva gingiva 48,8%, tonsilla 25,4%, mucosa labial 9,2%, palato dur e molle combinate 7,8%, mucosa buccal 5,4%, lingua 1,5%, pharynge 1,5%, e organo de email 0,76%.

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Primary Pulmonary Tumors of the Dog
A Report of Sixteen Cases

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MANY STUDIES have been carried out in recent years to evaluate the effect of environmental aerosols on the occurrence of pulmonary tumors in man. One approach to this problem has been to test the carcinogenic effects of tobacco smoke and industrial pollutants on the respiratory mucosae of laboratory animals. Little attention has been given to the presence of pulmonary tumors in domestic animals. Therefore, it seems of interest to extend this area of research to naturally occurring tumors in these species. For this purpose, the dog is uniquely suited as it is often kept as a pet under living conditions comparable to those of man, and it is allowed to live its entire life span, thus reaching the age where most cancers occur. The purposes of this study were: (1) to establish the incidence of canine pulmonary tumors in the collection of the Department of Veterinary Pathology, College of Veterinary Medicine, Ohio State University; (2) to determine if an increase of incidence has occurred in the last decade; (3) to describe their gross and histopathologic features; (4) to determine their site of origin in the respiratory tract; and (5) to investigate the possible influence of environment on the development of pulmonary neoplasms.

REVIEW OF LITERATURE

Monlux,[16] in 1952, reviewed the literature on pulmonary tumors in animals. He found 45 acceptable primary tumors of the lung in the dog and described 19 more tumors from the collection of the Royal Veterinary College in Stockholm, Sweden. Of the 64 canine tumors, 33 were columnar cell adenocarcinomas, seven were squamous carcinomas, one was a chondroma, one a lipoma, and 22 were simply classified as carcinomas.

The following cases have been reported since Monlux's review or were not included. Jenny,[11] in 1946, described an adenocarcinoma with squamous metaplasia in the left diaphragmatic lobe of a 7-year-old male Pointer. The tumor was the size of a child's fist and had caused progressive respiratory distress and emaciation.

Willis,[20] in 1948, described a bronchial adenocarcinoma with metastases to other parts of the lungs and lymph nodes in a 9-year-old English Setter.

Povar and Povar,[19] in 1950, reported a bronchial carcinoma in a 7-year-old female Collie, which had dyspnea and an occasional cough for one month. Radiographs revealed a mass involving the entire left diaphragmatic lobe. Necropsy revealed hydrothorax and a reddish gray mass with a necrotic center. Microscopically, acinar formation was evident. The cells were greatly variable in size and shape and had clear cytoplasm, vesicular nuclei, and frequent mitoses.

Stünzi,[26] in 1952, described two cases of canine bronchial carcinomas, one in a 4-year-old male Gordon Setter and the other in a 10-year-old female Cocker Spaniel. Both showed respiratory distress and extensive pulmonary osteoarthropathy of the long bones.

Ferri and Tausk,[6] in 1955, described 4 dogs with pulmonary epithelial tumors. Two of these, a 10-year-old male German Shepherd Dog and a 17-year-old male mongrel provided sufficient information to warrant a diagnosis of bronchiolar adenocarcinoma. The remaining 2 dogs were of uncertain origin.

Krahnert,[12] in 1954, reported a nodular tumor of both lungs in a 14-year-old male terrier. The dog was being treated for respiratory distress and emaciation. After 12 days of unsuccessful treatment, euthanasia was performed. Metastases were present in the bronchial and mediastinal nodes, liver, and kidneys. Microscopically, the tumor was a papillary adenocarcinoma believed to have originated in the bronchial mucous glands.

Dahme,[2] in 1956, described two cases. One occurred in a 12-year-old female Dachshund which developed nervous signs and emaciation. A diffuse carcinomatosis of all lobes with alveolar cell carcinoma had metastasized to the bronchial nodes, the heart, and the piriform lobe of the cerebrum. The second case, in an 8-year-old German Shepherd Dog, was characterized by severe dyspnea and radiographic density of the main bronchi. A diffuse alveolar cell carcinoma was present throughout the lungs. Metastases were found in the bronchial nodes, heart, and brain.

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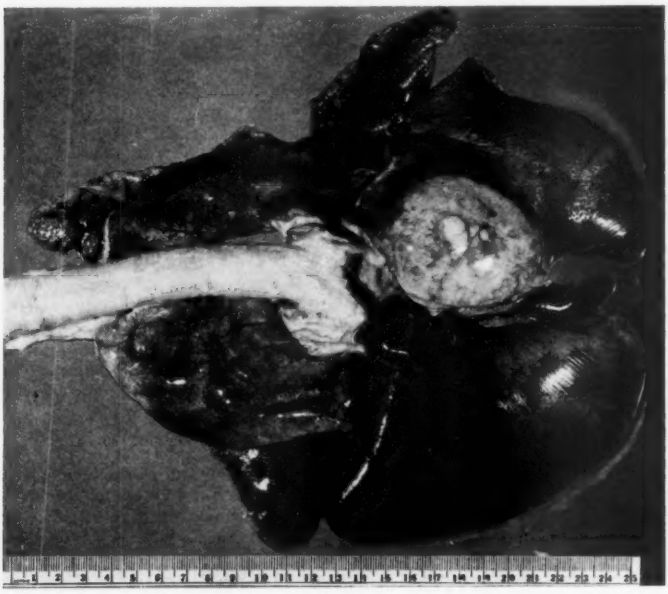

Fig. 1—Lung of dog 1 has an ovoid, white, bronchiolar adenocarcinoma in the dorsal portion of the right diaphragmatic lobe. Notice the generalized anthracosis.

Rooney,[20] in 1956, described a bronchiolar adenocarcinoma of the right lung in an 8-year-old female mongrel which had anorexia and a cough for two months. Microscopically, squamous transition was present.

Ten Thije, [27] in 1956, reported 22 canine primary pulmonary tumors, observed at 9,784 necropsies. Sixteen of 18 tumors metastasized. Microscopic studies were carried out on tumors from 18 dogs; 16 were adenocarcinomas, one was an undifferentiated carcinoma, and one was a mixed-cell tumor. Based on the pathology records from 1924 to 1954, it was postulated that the incidence of canine pulmonary tumors has increased in recent years, thus paralleling the findings in man.

Smith and Jones, [24] in 1957, found, in the collection of the Armed Forces Institute of Pathology, 28 squamous or anaplastic carcinomas, two sarcomas, and one hemangioma in the dog.

Sedlmeier and Dahme,[22] in 1958, described five tumors, representing an incidence of 0.24 per cent of all canine material examined. They were classified as adenocarcinomas and believed to have originated in the alveolar cells. The average age of the 5 dogs was 10.1 years.

MATERIAL AND METHODS

This study was based on the pathologic examination* of the pulmonary tissues collected during 9,882 canine necropsies performed in this department from July 1, 1928, to July 1, 1959. The habitats of the dogs were recorded as rural or urban.

Tissues were fixed in 10 per cent buffered isotonic formalin and stained routinely with hematoxylin and eosin. Special techniques used Mayer's mucicarmin, McManus' periodic acid-Schiff (PAS), and Gridley's elastin stains.

RESULTS

Dog 1 (Acc. No. 50756).—No history was available for dog 1.

Necropsy.—The right diaphragmatic lobe contained an ovoid, well-circumscribed, gray-white nodule, 6 by 5 cm., near the dorsal mediastinal border (fig. 1). In the remaining lung tissue, severe anthracosis was seen, but no nodules were found.

Histopathology.—The pulmonary nodule was a terminal bronchiolar adenocarcinoma. It had small square cells on an extremely delicate basement membrane consisting of a few strands of collagen around a single capillary. The cells had homogeneous eosinophilic cytoplasm with only a few vacuoles present and an uneven ragged outline toward the luminar side of the cell. The nuclei were large and vesicular with one or two nucleoli visible. Mitoses were not found. Atelectasis and a few scattered clusters of adenocarcinoma were present in the

* In cooperation with R. L. Farrell, D.V.M., Ph.D., and R. A. Griesemer, D.V.M., Ph.D., whose contributions are gratefully acknowledged

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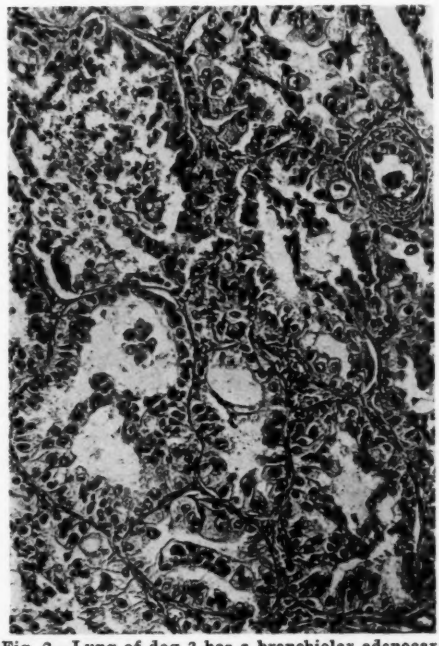

Fig. 2—Lung of dog 3 has a bronchiolar adenocarcinoma with an alveolar pattern. Large polygonal tumor cells are located on a delicate fibrous stroma. H & E stain; x 160.

surrounding alveoli, indicating direct extension via air passages.

Dog 2 (Acc. No. 5191).—Dog 2 was a 5½-month-old female English Setter from Columbus, Ohio.

Anamnesis.—The pup was brought in for distemper vaccination but, on clinical examination, early signs of distemper were already present. Two weeks later, the dog was readmitted because of conjunctivitis, diarrhea, and convulsions. During the next six weeks, the dog's condition deteriorated rapidly. Purulent nasal discharge, pneumonia, and continued convulsions were seen. Euthanasia was performed because the dog did not respond to prolonged therapy.

Necropsy.—The pup, weighing 18 lb., was emaciated and had a purulent nasal exudate and a rough haircoat. In the lungs was one firm white nodule aboue 1 cm. in diameter; areas of consolidation alternated with areas of emphysema.

Histopathology.—The terminal bronchiolar adenocarcinoma consisted of cuboidal cells of uniform size and shape, arranged in a papillary pattern on a thin, collagenous basement membrane. Many cross sections of the papillary projections appeared as detached rings of tumor epithelium cover-

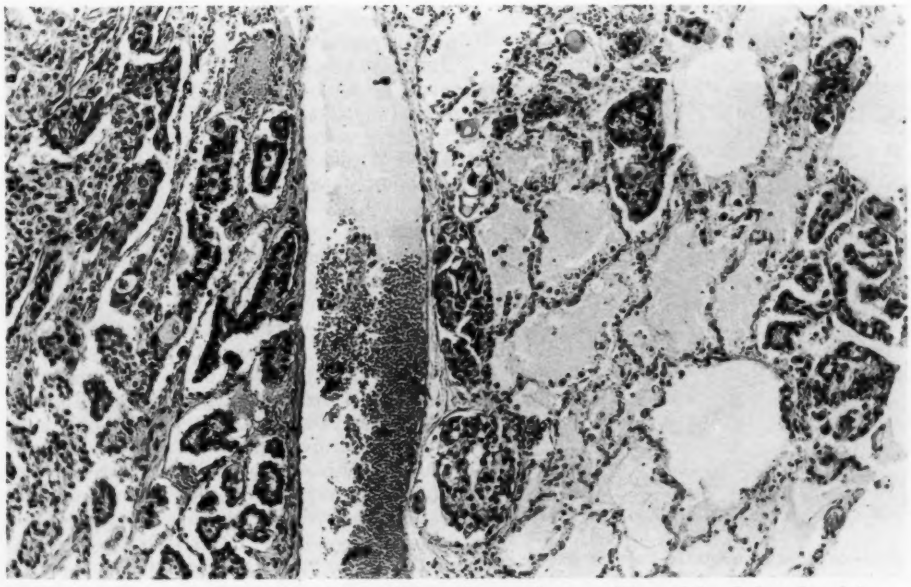

Fig. 3—Lung of dog 3 has a bronchiolar adenocarcinoma with peripheral invasion into surrounding alveoli. H & E stain; x 150.

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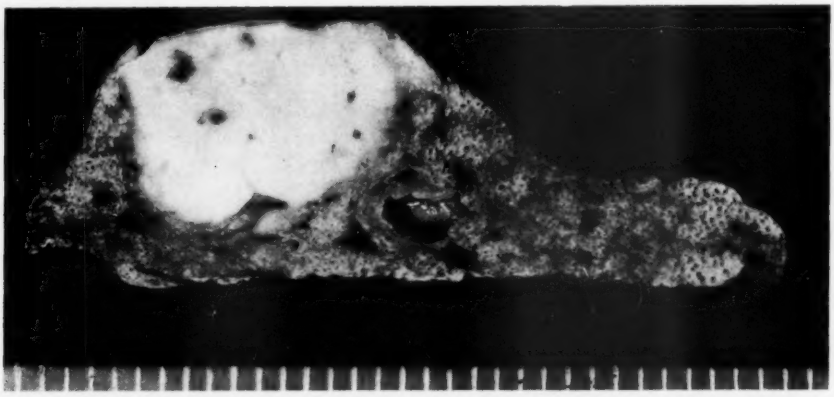

Fig. 4—The left diaphragmatic lobe of dog 5 with an ovoid, white, bronchiolar adenocarcinoma. The ruler at the bottom indicates millimeter divisions.

ing a central core of connective tissue stroma containing a vessel. The cytoplasm had a well-demarcated outline and it stained unevenly eosinophilic. The nuclei were large and centrally located, and had even chromatin distribution. Atelectasis was present in the surrounding lung tissue which was characterized by acute interstitial pneumonia, slight anthracosis, and occasional extension of neoplastic cells from the tumor mass.

Dog 3 (Acc. No. 52410).—Dog 3 was a 6-year-old male toy terrier.

Fig. 5—Right diaphragmatic lobe of dog 6 with a spherical, white, firm, well-delineated bronchiolar adenocarcinoma. Notice severe anthracosis in surrounding lung tissue.

Anamnesis.—The dog had been in poor health for an undetermined length of time with cough and weight loss. Treatment with antibiotics did not improve the condition.

Necropsy.—A pyramidal mass, measuring 5 cm. at the base, was present in the lung. The shape of the lobe was suggestive of its being either an apical or a cardiac lobe.

Histopathology.—A terminal bronchiolar adenocarcinoma was seen, with predominantly cylindrical cells lining collagenous alveolar septa. Some areas had distinct alveolar arrangements of large, polygonal, septal cells (fig. 2) with indistinct cellular borders, vesicular nuclei, and swollen nucleoli. Other areas had typical bronchiolar patterns with low cuboidal epithelium and small, dense nuclei (fig. 3). Their cytoplasm often appeared slightly foamy and vacuolated, but PAS and mucicarmin stains were negative for cytoplasmic mucus. Diffuse invasion of surrounding alveoli occurred at the entire periphery of the tumor (fig. 3), and clusters of tumor cells were present in pleural lymphatics.

Dog 4 (Acc. No. 55575).—Dog 4, from Columbus, Ohio, was a 10-year-old male Cocker Spaniel.

Anamnesis.—The dog was depressed, inappetent, and occasionally vomited. Respirations were 60 and the pulse, 90 per minute.

Necropsy.—The lungs were congested and the right diaphragmatic lobe had a firm, white, spherical nodule, 1 cm. in diameter.

Histopathology.—A terminal bronchiolar adenocarcinoma was diagnosed. The cuboidal cells were arranged in an alveolar pattern and, in many areas, were necrotic. The cells had dense eosinophilic cytoplasm without vacuole formation. Small, dense, and hyperchromatic nuclei alternated with larger vesicular forms, and only few mitoses were found.

Dog 5 (Acc. No. 5632).—Dog 5 was a 12-year-old female terrier from Delaware, Ohio.

Anamnesis.—The dog was depressed, anorectic, and had vomited for an unspecified period of time.

Necropsy.—Death was attributed to a fatty liver and relapsing pancreatitis which had resulted in pyloric stenosis by a ring of necrotic peripancreatic fat. As an incidental finding, a nodule, 2 by 2 cm., was found at the anterior margin of the left apical lobe (fig. 4).

Histopathology.—A terminal bronchiolar adenocarcinoma consisted of cuboidal cells forming an alveolar pattern. The nuclei of the tumor cells were toward the base of the cytoplasm which was often clear or slightly foamy in appearance. The PAS and mucicarmin stains did not reveal any cytoplasmic mucin. The tumor cells invaded the surrounding lung tissue without any evidence of encapsulation. In one section, a possible transition from the tall cylindrical cells of a small bronchiole (having ciliated and goblet-containing epithelium) to the tumor cells was demonstrated. The tall cylindrical cells in one end of the compressed bronchiole became metaplastic and they lost their regular palisaded arrangement. From this area, a long pedunculated strand of atypical cells extended into the lumen.

Dog 6 (Acc. No. 561229).—Dog 6 was an 11-year-old female mongrel from Columbus, Ohio.

Anamnesis.—The dog had a persistent cough which had not responded to antibiotic treatment. It tired easily upon exercise. A diagnosis of cardiac insufficiency was established clinically, and euthanasia was recommended.

Necropsy.—A soft, light-gray, ovoid mass, measuring 6 by 6 cm., replaced most of the left diaphragmatic lobe (fig. 5). The mass had a smooth surface and was covered by an intact pleura. Severe anthracosis was present throughout all lobes.

Fig. 6—Lung from dog 6 with a bronchiolar adenocarcinoma causing atelectasis of surrounding lung tissue to the right. H & E stain; x 165.

Histopathology.—A terminal bronchiolar adenocarcinoma was seen, with papillary rows of neoplastic cells on a delicate basement membrane. The cells were low cuboidal and were uniform in size, shape, and

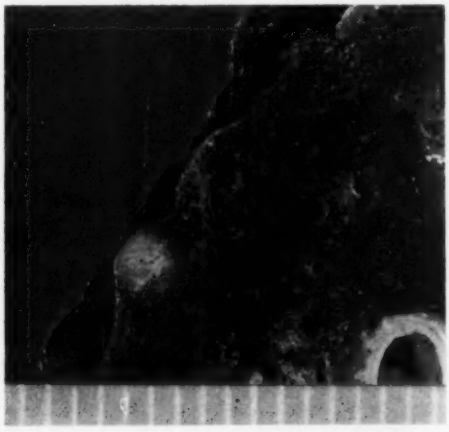

Fig. 7—Lung from dog 7 with a small, circumscribed, subpleural bronchiolar adenocarcinoma. The scale at the bottom indicates millimeter divisions.

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staining affinity. The nuclei were ovoid and hyperchromatic; mitoses were rare. The stroma was sparse, often consisting of a single vessel with its perivascular connective tissue. The tumor tissue gradually invaded the surrounding alveoli and resulted in compression atelectasis (fig. 6). A few focal areas of bronchiolar adenocarcinoma were present several millimeters away from the large primary tumor; no connections to it could be demonstrated in serial sections. By cutting numerous sections of the entire lung, one isolated subpleural area of bronchiolar adenocarcinoma was found in the right intermediate lobe. The neoplastic areas seemed to have originated by gradual metaplasia and anaplasia of normal terminal bronchioles. There was no evidence of lymphatic or hematogenous tumor embolism, and the bronchial lymph nodes did not contain any tumor cells.

Dog. 7 (Acc. No. B201).—Dog 7 was an 8-year-old male Boston Terrier from Lima, Ohio.

Anamnesis.—The dog was submitted for removal of a large subcutaneous liposarcoma. As complete surgical removal was not possible, euthanasia was recommended.

Necropsy.—As an incidental finding at necropsy, a small white nodule, 2 mm. in diameter, was found in a subpleural area of a cardiac lobe (fig. 7). Pronounced anthracosis was present throughout the entire lung.

Histopathology.—A terminal bronchiolar adenocarcinoma was present. It consisted of uniform cuboidal cells having a slight amount of eosinophilic homogeneous cytoplasm and ovoid hyperchromatic nuclei without mitoses. Periodic acid-Schiff stain did not stain material in the cytoplasm of the tumor cells, while much PAS-positive material was evident in the bronchial epithelium deeper in the same section. No encapsulation was evident and isolated clumps of tumor cells were found in the neighboring alveoli. The overlying pleura was slightly elevated and atrophied but appeared intact (fig. 8).

Dog 8 (Acc. No. B1252).—Dog 8 was a 14-year-old spayed German Shepherd Dog from Columbus, Ohio.

Anamnesis.—The dog was submitted to the clinic because of weakness and pain in

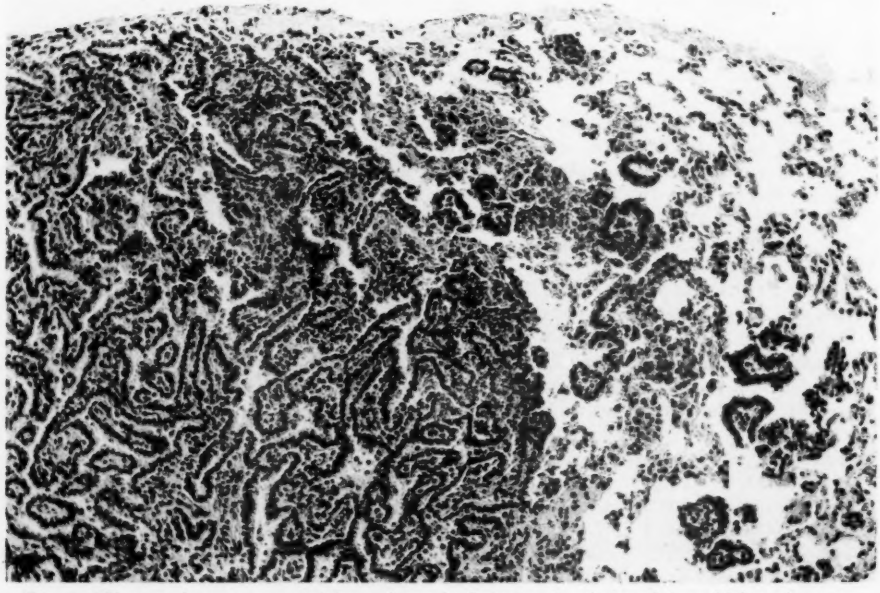

Fig. 8—Microscopic appearance of lung shown in figure 6. A subpleural bronchiolar adenocarcinoma is present with extension into surrounding alveoli. H & E stain; x 160.

the hindlegs due to chronic osteoarthritis of both stifle joints.

Necropsy.—As an incidental finding in the respiratory system, a white, rather soft, well-circumscribed nodule measuring 2.5 by 2.0 cm. was present in the medial side of the right diaphragmatic lobe. Diffuse anthracosis was present in all lobes of the lung and the bronchial nodes. Numerous small, hard, mineralized nodules, 1 to 3 mm. in diameter, were scattered throughout the lung; they were interpreted grossly as healed lesions of histoplasmosis.

Histopathology.—A bronchiolar adenocarcinoma was diagnosed. It consisted of single rows of large cells forming alveoli and located on thin fibrous septa. The cells were square or slightly rectangular in outline with well-demarcated cytoplasmic borders which, on the luminal side, occasionally had small projections containing minute vacuoles. These were seen to contain PAS-positive and mucicarmin-positive material, as did the corresponding lumina. The nuclei of the tumor cells were vesicular, with prominent nucleoli. Mitotic figures were not found. Necrosis and mineralization were seen and many clefts were present in the central part of the tumor. They were believed to have been caused by fatty acid crystals. The tumor cells extended into neighboring alveoli. Neither vascular invasion nor metastases were demonstrated.

Dog 9 (Acc. No. B1305).—Dog 9 was a 20-year-old male Beagle from Canal Winchester, Ohio.

Anamnesis.—The dog was submitted for euthanasia because of old age, arthritis, and a large ulcerating perianal gland tumor.

Necropsy.—Four different tumors were found: a perianal gland adenoma, a Leydig cell tumor, a seminoma, and a subpleural tumor of the right cardiac lobe. The lung tumor measured 2.5 by 2.0 cm. and was round, firm, white, and well demarcated. Anthracosis and some diffuse fibrosis and loss of elasticity were present.

Histopathology.—A terminal bronchiolar adenocarcinoma was diagnosed. It consisted of uniform cuboidal cells with hyperchromatic, ovoid nuclei situated in the basal part of eosinophilic cytoplasm (fig. 9). Mitoses were extremely rare. Most tissue had only one row of cuboidal cells lining the lumina, but occasionally two cell layers were present. Much necrosis was seen in the center of the tumor, some of which had

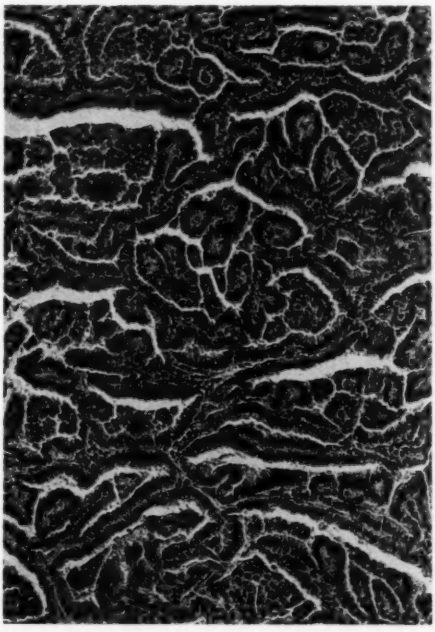

Fig. 9—Lung of dog 9 with a bronchiolar adenocarcinoma depicting a papillary arrangement of uniform cuboidal cells. H & E stain; x 160.

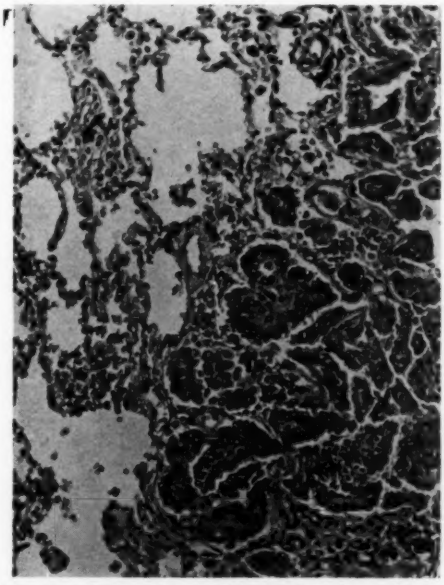

Fig. 10—Lung of dog 10 illustrating the periphery of the bronchiolar adenocarcinoma without any evidence of encapsulation. H & E stain; x 165.

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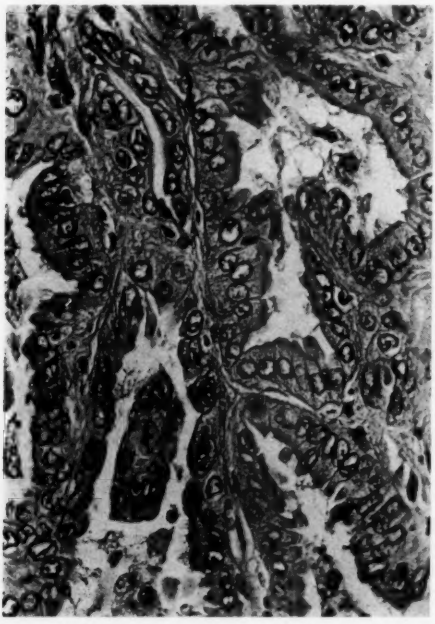

Fig. 11—Lung from dog 10 with a bronchiolar adenocarcinoma showing ciliated cylindrical tumor cells with vesicular nuclei on a delicate fibrous stroma. H & E stain; x 475.

undergone hyalinization or mineralization.

Dog 10 (Acc. No. C200).—Dog 10 was a 12-year-old male Springer Spaniel from Columbus, Ohio.

Anamnesis.—The animal was caught in a flood five days before being submitted and developed hematuria and depression.

Necropsy.—Anthracosis was seen in all lobes of the lungs and in the bronchial lymph nodes. The right diaphragmatic lobe had in its posterior ventral border a firm, white, well-circumscribed nodule which measured 3 by 2 cm.

Histopathology.—A bronchiolar adenocarcinoma was diagnosed. No encapsulation was evident (fig. 10) and areas immediately surrounding the tumor were seen to contain islets of tumor cells, free both in alveoli and in the peribronchial and pleural lymphatics. Cylindrical cells of uniform size and shape lined thin fibrous septa (fig. 11). Their nuclei were ovoid, vesicular, and without mitotic figures. The cytoplasm was homogeneously eosinophilic and had distinct luminal borders which occasionally were ciliated. There were PAS-positive granules in the cytoplasm of both hyperplastic and neoplastic bronchiolar epithelium. Metastases were found in pleural lymphatics and in one bronchial lymph node.

Dog 11 (Acc. No. C347).—Dog 11 was a 7-year-old female Cocker Spaniel from Tampa, Florida.

Anamnesis.—The dog was obtained for use in experimental thoracic surgery. On incision of the left thoracic wall, a mass was found involving two thirds of the left diaphragmatic lobe. The dog was unsuitable for further study and was killed. There had been no history of previous illness or the presence of tumors.

Necropsy.—The dog weighed 35 lb. At necropsy, in addition to the lung tumor, a small nodule was found in the spleen. Histologically, it was found to be an area of lymphoid hyperplasia. A mesenteric node was found to be enlarged, but histologic examination of it revealed only lymphoid hyperplasia.

Histopathology.—A bronchiolar adenocarcinoma was diagnosed. Papillary neoplastic structures with large lumina (fig.

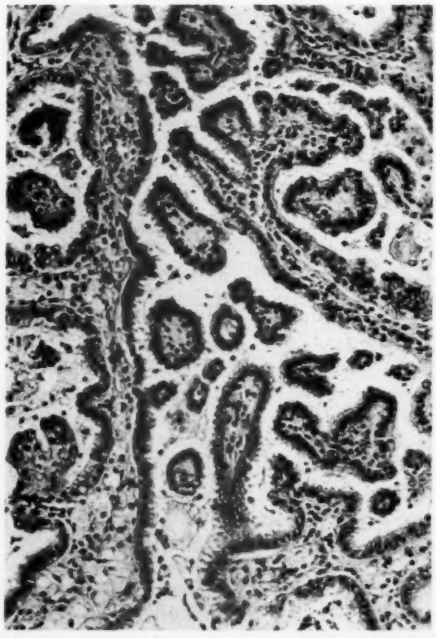

Fig. 12—Lung of dog 11 with a bronchiolar adenocarcinoma showing a papillary arrangement of cuboidal cells. H & E stain; x 165.

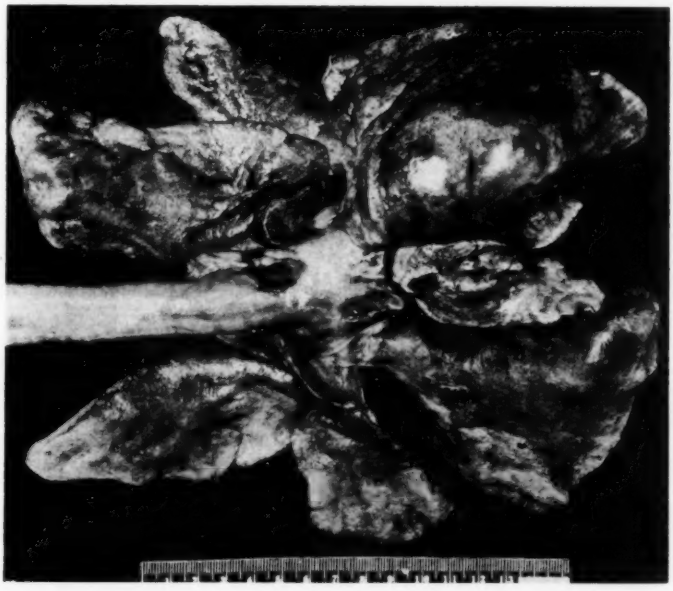

Fig. 13—Lung of dog 13, depicting a large, ovoid, pleura-covered bronchial carcinoma of the right diaphragmatic lobe. Much anthracosis of the lung and bronchial nodes is present.

12) alternated with more dense areas. The tumor cells were mostly cuboidal with a few areas of cylindrical cells. The nuclei were large and ovoid and had evenly distributed chromatin. Mitotic figures were not seen. The cytoplasm was homogeneously eosinophilic without any vacuoles. The cytoplasmic borders were irregular and poorly outlined. Lymphatic or vascular invasion was not seen.

Dog 12 (Acc. No. C693).—Dog 12 was a 10-year-old female Boxer from Cincinnati, Ohio.

Anamnesis.—For four months, the dog had clinically atypical chronic pneumonia, partial anorexia, weight loss and normal body temperature. There was no response to medication. The veterinarian made a tentative diagnosis of granulomatous pneumonia, possibly histoplasmosis or tuberculosis.

Necropsy.—Approximately 90 per cent of the lung tissue was transformed into a fairly uniform grayish white consolidated mass which did not collapse after removal from the chest cavity. All lobes were diffusely involved and no distinct nodules were found on palpation.

Histopathology.—The bronchiolar adenocarcinoma consisted of large cuboidal and cylindrical cells arranged in a single layer

Fig. 14—Bronchus of dog 13, illustrating hyperplasia and squamous metaplasia of the mucosa. H & E stain; x 165.

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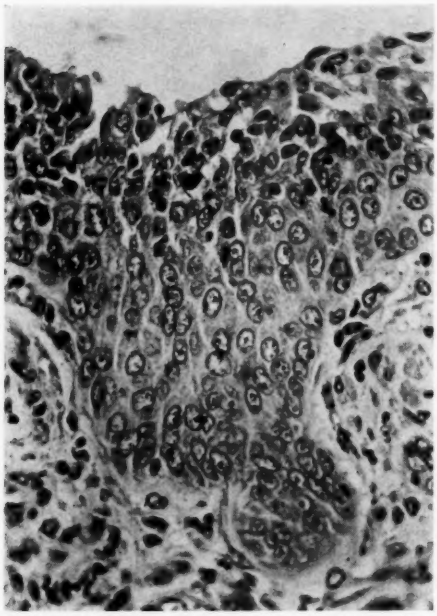

Fig. 15—Bronchus of dog 13, illustrating squamous metaplasia of mucosa. H & E stain; x 475.

on a delicate fibrous stroma. The tumor cells maintained this acinar arrangement throughout most of the lung with only occasional formation of solid islands. The cells were slightly eosinophilic and vacuolated, with distinct borders which often had eosinophilic apices extending into the acinar lumina. The nuclei were vesicular, and only occasional mitoses were encountered. Mucicarmin and PAS stains showed a few slightly positive droplets in the cytoplasm of the tumor cells, while the secretion encountered in the lumina was strongly positive.

Dog 13 (Acc. No. 541752).—Dog 13 was a 12-year-old spayed Standard Poodle from Columbus, Ohio.

Anamnesis.—The dog had a cough and mucopurulent expectorate for three months, which were attributed to a chronic bronchitis. Progressive weakness with difficult locomotion and stiffness in the hindlegs had been noticed for six weeks.

Necropsy.—An ovoid, firm, gray nodule, measuring 7 by 5 cm., was present in the mid-dorsal part of the right diaphragmatic lobe (fig. 13). Several smaller nodules were seen throughout the remaining lobes. Severe anthracosis was seen in the entire lung.

Histopathology.—A bronchial squamous carcinoma was seen originating in small bronchi. There was transition from normal, cylindrical, epithelial cells to large hyperplastic and anaplastic cells having vesicular nuclei and no nuclear polarity (fig. 14, 15). There was piling of atypical cells which formed large masses filling out the lumen and extending into the neck and fundus of bronchial glands. Occasionally, invasion through the peribronchial fibroblastic membrane into surrounding alveoli was seen. Numerous small arteries and veins contained thrombi and tumor emboli. Many peribronchial lymphatics were completely occluded with tumor cells. Serial sections revealed extension of anaplastic bronchial epithelium for at least 4 mm. along one bronchus. The tumor cells were large and squamous with faint eosinophilic cytoplasm and huge vesicular nuclei with frequent mitoses (fig. 16). Metastases were present in bronchial and mediastinal lymph nodes.

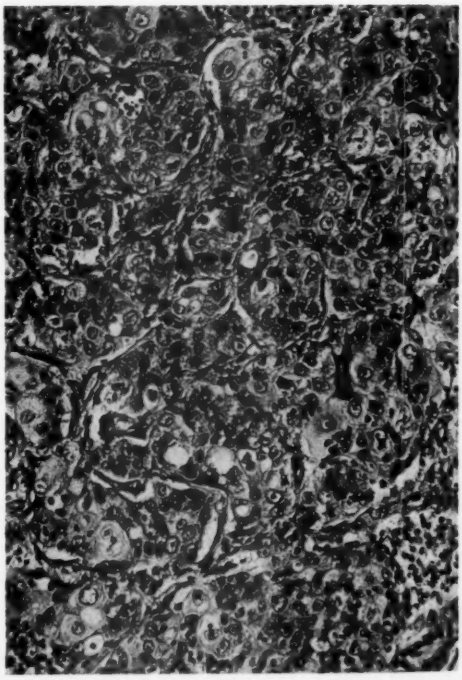

Fig. 16—Lung of dog 13 with bronchial squamous carcinoma showing diffuse infiltration of alveoli with large, tumorous squamous cells. H & E stain; x 160.

Dog 14 (Acc. No. A402).—Dog 14 was a 10-year-old female Cocker Spaniel from Columbus, Ohio.

Anamnesis.—The dog was anorectic for several days and had severe dyspnea which had become progressively worse. Radiographs of the thorax revealed a tumor of the left lung. A biopsy revealed a bronchial carcinoma and, because a poor prognosis was given, euthanasia was recommended.

Necropsy.—A large, white, well-circumscribed tumor, measuring 6 by 5 cm., was found in the ventroposterior portion of the left diaphragmatic lobe, which also contained a small, round nodule, 8 by 6 mm., near its hilus (fig. 17). Two smaller nodules, which measured about 5 mm. in diameter, were present in the left cardiac and the right apical lobes. The pleura covering the left diaphragmatic lobe was thickened and had torn, fibrous adhesions. Pronounced anthracosis was present.

Histopathology.—A bronchial carcinoma was diagnosed. Transition from normal bronchial epithelial cells to hyperplastic and neoplastic cells could be observed (fig. 18). The epithelium became irregular, with marked thickening, palisading, and folding. Diffuse infiltration of lung tissue was present, with clumps of small pleomorphic and atypical cells containing hyperchromatic nuclei, several mitoses, and sparse, ill-defined cytoplasm. The pleura covering the tumor was invaded by tumor cells and extension of the tumor via bronchial lumina was evident. No metastases were found in the bronchial nodes.

Dog 15 (Acc. No. C2).—Dog 15 was a 5-year-old female Beagle from West Jefferson, Ohio.

Anamnesis.—The dog had been sick for four weeks with anorexia, difficult breathing, and a body temperature of 102.5 F. Antibiotics and supportive therapy resulted in the return of normal body temperature; dyspnea persisted. A slight amount of free blood was present in both nostrils for three days prior to euthanasia.

Necropsy.—A large, raised, red, consolidated area was present in the anterior margin of the left diaphragmatic lobe. Grossly, the surrounding lung tissue had greatly distended pleural lymphatics, appearing as whitish threads (fig. 19). Many infarcts were present in kidney and spleen.

Histopathology.—A bronchial squamous carcinoma was diagnosed. Many smaller bronchi and bronchioles showed anaplastic changes in their mucosae with piling of nuclei, loss of polarity, and hyperchromatism. In some areas, the anaplastic change could be followed down into the necks of the bronchial mucous glands (fig. 20). There were PAS-positive droplets in the cytoplasm of the tumor cells. Lymphatics, veins, arterioles, and medium-sized arteries throughout the lungs contained tumor emboli. Metastases were seen in the pleural lymphatics (fig. 21), the bronchial nodes, the kidneys, and the spleen. The grossly affected lung area in the anterior margin of the left diaphragmatic lobe was the only site where anaplasia could be demonstrated in bronchial epithelium. As an incidental finding, several empty egg shells of *Paragonimus kellicotti* were found in the tumorous lung area.

Dog 16 (Acc. No. A1772).—Dog 16 was a 13-year-old female Springer Spaniel from Columbus, Ohio.

Anamnesis.—The dog was being treated for anorexia and a urinary bladder condition. It expired under anesthesia while it was being prepared for radiography.

Necropsy.—Examination revealed an ulcerating squamous carcinoma of the blad-

Fig. 17—Lung of dog 14 depicting one large and one small nodule (arrow) of bronchial carcinoma in the diaphragmatic lobe.

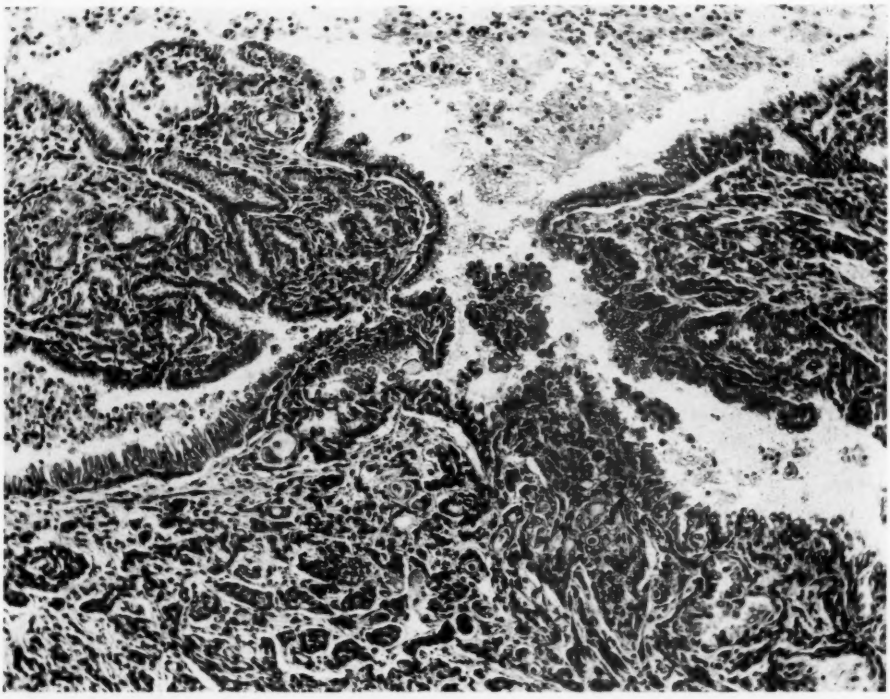

Fig. 18—Bronchus of dog 14 demonstrating mucosal hyperplasia (above) and neoplasia (below) with clumps of small, highly anaplastic tumor cells. H & E stain; x 170.

der with multiple metastases to sublumbar lymph nodes, adrenals, liver, kidneys, heart, and lungs. Small, firm, white pulmonary nodules ranged in size from 1 to 5 mm.; one proved to be a bronchial adenoma. On microscopic examination of the section, it appeared circular and was 5 mm. in diameter.

Histopathology.—A spherical bronchial adenoma was surrounded by a rim of atelectatic alveoli, which occasionally showed extension of neoplastic bronchial epithelium. Areas of normal ciliated and goblet-bearing bronchial epithelium could be traced into the greatly hyperplastic and papillary epithelium containing both vesicular and hyperchromatic nuclei (fig. 22). The surrounding lung tissue had many intravascular emboli consisting of two to eight squamous cells originating in the urinary bladder carcinoma.

DISCUSSION

The diagnosis of bronchial adenocarcinoma was used as a designation for this uniform group of 12 tumors which were believed to have originated in the low cuboidal epithelium of the bronchioles. This term was preferred to others used to describe tumors of similar morphology; namely, pulmonary adenomatosis and alveolar cell carcinoma. The term, "pulmonary adenomatosis," is less desirable for this type of canine tumor, as it already has a specific meaning in veterinary pathology denoting a bilateral, multifocal adenomatous process, possibly of infectious nature, as described in sheep by Dungal [4] and in chinchillas by Helmboldt *et al.*[7] In recent years, Herbut [8,9] and Hutchison [10] have made strong arguments in favor of the terminal bronchiolar epithelium as the origin of this neoplasm. Three reports were found in which similar studies were carried out on pulmonary tumors in animals. Krahnert [14] considered the bronchial glands to be the cell of origin for the vast majority of pulmonary carcinomas reported in animals. He stated that of 83 animals sufficiently described histologically, 64 had the

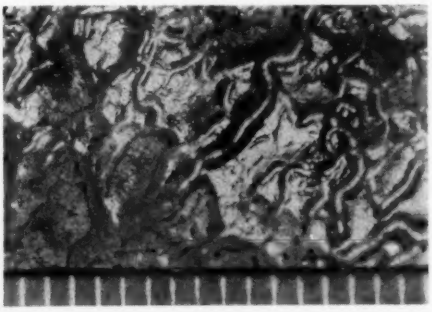

Fig. 19—A gross photograph of the pleural surface of a diaphragmatic lobe from dog 15, showing white, tumorous lung tissue with greatly distended pleural lymphatics which are outlined by phagocytized carbon pigment. The ruler at the bottom indicates millimeter division.

bronchial glands as the site of origin for a pulmonary carcinoma.

Sedlmeier and Dahme,[22] on the basis of a study of five canine pulmonary adenocarcinomas and various chronic pneumonias, concluded that the tumors originated in the alveolar septal cells. Sjolte,[23] in a report of ten canine primary pulmonary tumors, depicted (fig. 10) a transition from normal bronchiolar epithelium to an adenocarcinoma.

The material in the present study confirms his observation of bronchiolar origin. The 12 tumors, classified as bronchiolar carcinomas, had sufficient morphologic and biologic similarity to suggest a common cell of origin. If one considers the subpleural location of the tumor of dog 7 (fig. 6, 7, and 24), measuring 0.2 by 0.2 cm., it will be seen that it is not anatomically possible for this tumor to originate in the bronchial glands, as no bronchus large enough to have bronchial glands is present in the extreme periphery of the lung. The peripheral location of the bronchiolar adenocarcinoma was found to be a constant feature in the dog (fig. 23, 24). On the basis of our findings and those of Herbut, Hutchison, and Sjolte, and because of the morphologic similarity to normal bronchiolar epithelium, the possibility of origin in bronchial glands was excluded.

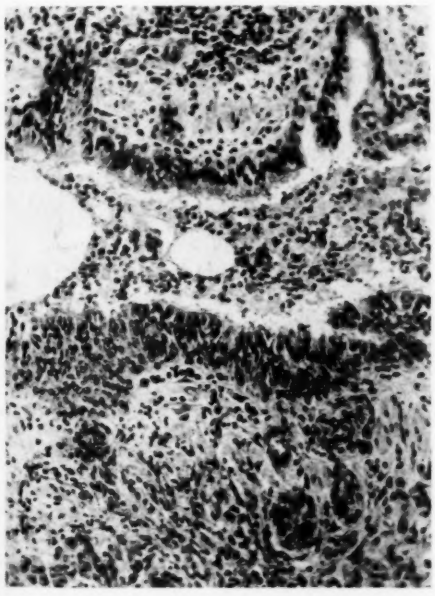

Fig. 20—Bronchus from dog 15 showing relatively normal mucosa above and metaplastic mucosa below. H & E stain; x 165.

Fig. 21—Lung from dog 15 depicting a distended pleural lymphatic containing a tumor embolus. H & E stain; x 165.

The peripheral, subpleural origin, the uniformity of the tumor cells (either cuboidal or cylindrical), the arrangement in a papillary pattern, the delicate stroma, and the rarity of metastases were the characteristic features of this neoplasm in the dog. Certain differences exist between it and its counterpart in the human lung. In

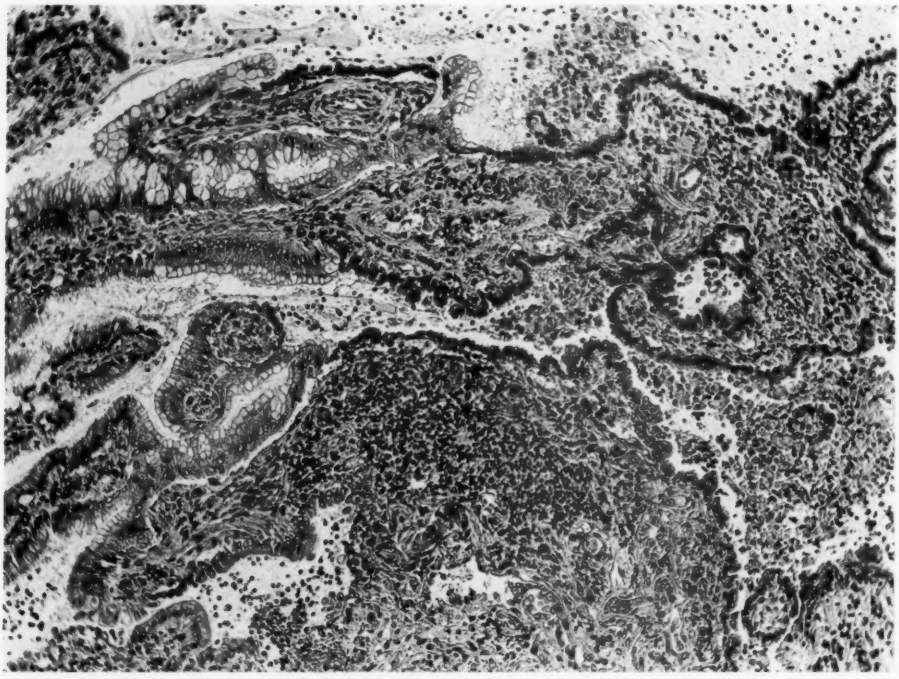

Fig. 22—Bronchus of dog 16 with a bronchial adenoma showing folding, hyperplasia, and metaplasia of the mucosa. H & E stain; x 170.

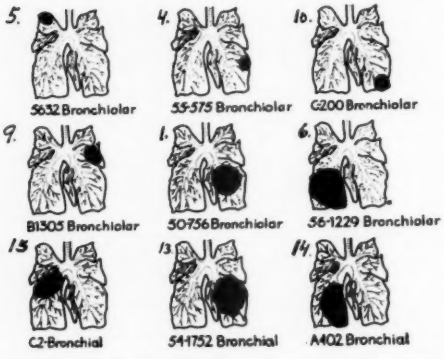

Fig. 23—A drawing of six bronchiolar adenocarcinomas (above) and 3 bronchial carcinomas (below) where location and size were known. The tumors are arranged according to their size. Notice the predominance of a peripheral location of the bronchiolar tumors, as opposed to the more hilar location of the bronchial carcinomas. The numbers in the upper left corners refer to the case numbers and the numbers below to accession numbers of the dogs.

the latter, peripheral origin and the characteristic cuboidal cells are present. According to Ewing's classification,[5] the tumor in man is found either as a multiple nodular type or as a diffuse infiltrating type. The human bronchiolar adenocarcinoma metastasizes in about half of the cases, while we found only 1 dog with metastasis. Other canine cases with which a comparison can be made were presented by Monlux.[16] From his description of individual cases, the following information could be extracted. Out of 19 cases, 14 had columnar epithelium and, of these, seven were found as solitary nodules and seven were of the multiple or diffuse type. Of the solitary type, only one metastasized and had a pseudostratification of its tall columnar cells, possibly indicating a histologic type different from the bronchiolar cells. Among the latter seven cases, six had metastases. Unfortunately, the illustrations of cell morphology were not adequate to allow further conclusions as to the cell of origin. Only dog 6 in our study had more than one neoplasm, sug-

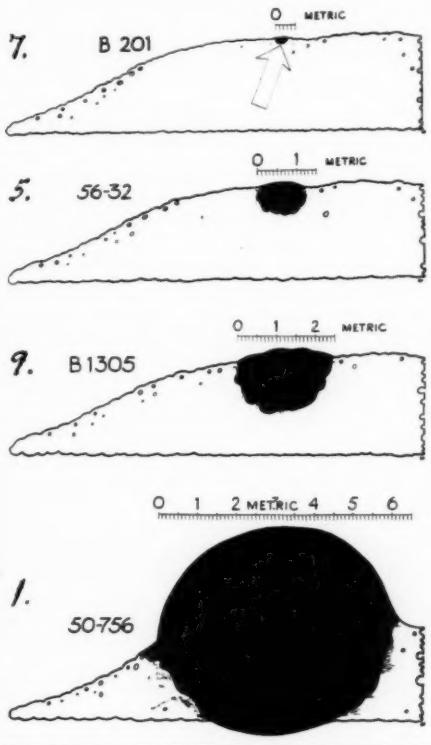

Fig. 24—A diagrammatic drawing of the progression in size of bronchiolar adenocarcinomas, as illustrated by four tumors in this study. The numbers in the upper left corner indicate the case numbers and the accession numbers.

gesting a multicentric origin. In addition to a large mass in the left diaphragmatic lobe, a small subpleural nodule, about 1 mm. in diameter, was present in the intermediate lobe. This finding was indicative of a multicentric origin rather than a metastatic process, which was one of the characteristic features of some of the canine cases described by Monlux[16] and of the human bronchiolar adenocarcinoma.[28] Only dog 12 had a gross picture that is compatible with the diffuse infiltrating type encountered by Monlux and which is common in man.

Two dogs, 13 and 15, were found to have primary squamous cell carcinomas of almost identical morphology. By serially sectioning blocks of selected lung areas, it was possible in both cases to demonstrate hyperplasia and metaplasia of the epithelium in small bronchi (fig. 13, 14, 20, and 21). The tumor cells were large and squamous with an abundance of eosinophilic cytoplasm and vesicular, nonpolar nuclei. The cell masses obliterated the lumina of bronchial glands when present and invaded the surrounding fibroelastic membranes and alveoli. Numerous tumor emboli were present in blood vessels and lymphatics in both peribronchial connective tissue and in the pleura. Metastases occurred in the bronchial nodes, spleen, and kidneys.

The two tumors in dogs 14 and 16 were of greatly different sizes but had the similar microscopic features of a bronchial tumor. The first was diagnosed as an anaplastic bronchial carcinoma consisting of tall cylindrical cells mixed with areas of small anaplastic cells; the second was designated a bronchial adenoma. In both, transition from tall columnar pseudostratified bronchial epithelial cells to tumor cells could be seen (fig. 17, 23). In dog 14, the tumor attained considerable size and a higher degree of anaplasia. Many areas had a highly pleomorphic, small-cell type with no tendency to acinar pattern. The morphology of these areas was compatible with the so-called oat-cell or small-cell carcinoma of

TABLE 1—Incidence of Canine Primary Pulmonary Tumors in the Collection of Necropsy Material at Ohio State University, July 1, 1928 to July 1, 1959

Year	Canine necropsies	No. of canine pulmonary tumors
1928–1929	365	0
1929–1930	356	0
1930–1931	283	0
1931–1932	274	0
1932–1933	360	0
1933–1934	342	0
1934–1935	336	0
1935–1936	404	0
1936–1937	239	0
1937–1938	232	0
1938–1939	245	0
1939–1940	220	0
1940–1941	143	0
1941–1942	253	0
1942–1943	252	0
1943–1944	425	0
1944–1945	350	0
1945–1946	242	0
1946–1947	280	0
1947–1948	219	0
1948–1949	268	0
1949–1950	236	0
1950–1951	220	2
1951–1952	202	1
1952–1953	217	0
1953–1954	270	0
1954–1955	484	1
1955–1956	502	2
1956–1957	432	2
1957–1958	276	2
1958–1959	355	6
Total	9,282	16

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the human lung, which is a frequent histologic type of bronchial carcinoma in man.

Of the 103 canine pulmonary tumors described in the literature, 63 were diagnosed as adenocarcinomas of either columnar or alveolar cell type, while only three were diagnosed as squamous cell carcinomas. A number of reports, however, contained only a diagnosis of pulmonary carcinoma and did not furnish sufficient pictorial or descriptive material to allow any conclusions as to histologic type or cell of origin. From the information available, it can be concluded that the most common primary tumor of the canine lung is bronchiolar adenocarcinoma and that it accounts for approximately three quarters of all primary lung tumors of the dog. This tumor is characteristically a solitary, peripherally located, well-circumscribed nodule with uniform cuboidal epithelium located on a septum-like stroma. It is an expansively growing neoplasm that only rarely metastasizes. The incidence of bronchiolar adenocarcinoma in man is much lower than in the dog, comprising only about five per cent of all lung cancers.[15,18] It is of interest that no primary mesenchymal tumors were found in the lungs of dogs in this study.

An increase in incidence of primary pulmonary tumors of the dog was seen by Ten Thije [27] in material from Holland, and suggested by Cohrs [1] in Hanover, Germany, and by Sjolte [23] in Copenhagen, Denmark. Krahnert,[12,13] from Leipzig, Germany, and Monlux,[16] in his survey of Swedish material, did not demonstrate such an increase. The material presented (table 1) is suggestive of an increase of canine pulmonary carcinomas in the last decade. Whether this is a real or an apparent increase is difficult to evaluate because of the rapidly growing interest in the diseases of dogs, with more complete pathologic examinations as a result. The numerical increase, particularly during the time of this study (1958 to 1959), might well reflect an increased awareness and interest in this tumor which, in turn, resulted in a more diligent search for it. A similar apparent increase in incidence, found in other neoplastic as well as non-neoplastic diseases of the dog, was unequivocally due to better diagnostic facilities. Among 9,282 dogs necropsied in this department during the past 31 years, 16 pulmonary tumors were found—an incidence of 0.17 per cent. From table 1, it can be seen that all 16 tumors were found within the last nine years. This is similar to the findings of Sedlmeier and Dahme,[22] who found the incidence of canine pulmonary carcinomas to be 0.24 per cent. Stünzi [25] found the incidence to be 0.5 per cent among 6,325 canine necropsies performed in Stockholm, Sweden. Dobberstein [3] found the percentage of pulmonary tumors to be 4.5 per cent of all malignancies in the dog.

The presence of anthracosis in the lungs and bronchial lymph nodes was consistent in the dogs studied. An attempt was made to establish the relationship between the degree of anthracosis and the environment of the dog. It was found that dogs 5 and 9, from rural areas, showed as much pulmonary anthracosis as several of the city dogs. It must be remembered, however, that the ages of those 2 dogs were 12 and 20, respec-

TABLE 2—Sixteen Primary Pulmonary Tumors of Dogs Diagnosed in the Department of Veterinary Pathology, Ohio State University, from July 1, 1928 to July 1, 1959

Dog No.	Acc.* No.	Breed	Sex	Age (yr.)	Habitat	Size (cm.)	Site (lobe of lung)	Signs	Metastasis	Cytology
1	50756	.....	....	..	.....	5 x 5	Rt. diaphr.	.....	.....	Cuboidal
2	5191	English Setter	F	.5	Urban	1 x 1	.....	None	None	Cuboidal
3	52410	Toy terrier	M	6	.....	5 x 5	.....	Cough	Pleura	Cuboidal
4	55575	Cocker Spaniel	M	10	Urban	1 x 1	Rt. diaphr.	None	None	Cuboidal
5	5632	Terrier	F	12	Rural	1 x 1	Lt. diaphr.	None	None	Cuboidal
6	561229	Mongrel	F	11	Urban	6 x 6	Rt. diaphr.	Cough	None	Cuboidal
7	B201	Boxer	M	8	Urban	.2 x .2	Cardiac	None	None	Cuboidal
8	B1252	German Shepherd Dog	SF	14	Urban	3 x 2	Rt. diaphr.	None	None	Cuboidal
9	B1305	Beagle	M	20	Rural	3 x 2	Rt. cardiac	None	None	Cuboidal
10	C200	Springer Spaniel	M	12	Urban	3 x 3	Rt. diaphr.	None	Br. node	Cuboidal
11	C347	Cocker Spaniel	F	7	Urban	.....	Lt. diaphr.	None	None	Cuboidal
12	C693	Boxer	F	10	Urban	.....	Diffuse	Dyspnea	None	Cuboidal
13	541752	Poodle	SF	12	Urban	3 x 3	Rt. diaphr.	Cough	Br. node	Squamous
14	A402	Cocker Spaniel	F	10	Urban	6 x 5	Lt. diaphr.	Dyspnea	Pleura	Anaplastic
15	C2	Beagle	F	5	Urban	.....	Lt. diaphr.	Dyspnea	Viscera	Squamous
16	A1772	Springer Spaniel	F	13	Urban	.5 x .5	.....	None	None	Columnar

* The first two digits denote year of necropsy, except where preceded by a letter (A = 1957, B = 1958, C = 1959). SF = spayed female; Br. = bronchial.

tively, a much greater age than the average for the city dogs.

The environment of the dogs with lung tumors was investigated by contacting the owners. There were 14 dogs with a known background; 12 were from urban areas and only 2 were country dogs (table 2). To evaluate the significance of this distribution, the location of the homes of a random sample of canine accessions to this department was determined and found to be of a ratio of 5 urban to 1 rural. This material failed to reveal a significant difference in the incidence of lung tumors between city dogs and country dogs. However, future studies of a larger number of cases may demonstrate such a difference.

The dogs in this study have a wide age range of 6 months to 20 years, with the mean and median age coinciding at 10 years. Of the two extreme ages (6 months and 20 years), the youngest appeared correct on further checking of the dog's clinical history and body weight. The oldest dog was born and raised on a farm near Columbus and the owner was able to provide the birth date. The mean age of 10 years was similar to that of 36 dogs of known age studied by Monlux. He found the average age to be 9.2 years, with the youngest ages being 14 and 30 months. The number of cases available was too small to see if any age difference occurred in the different histologic types of pulmonary carcinomas.

The sex distribution was 9 females to 5 males, which did not differ significantly from the sex distribution of the canine population received at the College of Veterinary Medicine, Ohio State University, which, in random sampling, was 270 females to 230 males. Monlux found a ratio of 19 males to 15 females among all dogs in which the sex was indicated.

The breed distribution was not of any significance because of the small number of dogs represented. There were 3 Cocker Spaniels, 2 terriers, 2 Boxers, and 2 Beagles, an incidence compatible with the normal canine population. In this area, in 1956–1957, the Cocker Spaniel was the breed most frequently found, accounting for 19 per cent of all dogs.[17] The terrier-type mongrel (11%) was second, the Collie was third, the Beagle fourth, and the Boxer fifth.

SUMMARY

A search of the literature revealed descriptions of 103 canine primary pulmonary tumors, 63 of which were classified as adenocarcinomas. In this report, 16 more primary pulmonary carcinomas are added. They were diagnosed following 9,282 necropsies of dogs during the 31-year period from July 1, 1928, to July 1, 1959. On the basis of their histologic structure and the site of origin, they were classified as bronchiolar adenocarcinomas,[12] bronchial carcinomas,[3] and bronchial adenoma.[1]

The bronchiolar adenocarcinomas of the dog appeared to have their primary focal origin in the periphery of the lung. Histologically, they had a single row of cuboidal cells in a papillary arrangement on thin connective tissue septa.

The bronchial carcinomas were found to originate in small secondary and tertiary bronchi, the mucosa of which was characterized by hyperplasia and squamous metaplasia. Two of the bronchial carcinomas were found to have squamous transition, while the third was of the undifferentiated small-cell type. All three bronchial carcinomas had metastasized, but only two of the 12 bronchiolar adenocarcinomas showed lymphatic invasion and bronchial node metastasis. The bronchiolar adenocarcinoma constituted three fourths of all primary pulmonary neoplasms studied.

The incidence of pulmonary neoplasms in necropsy material was approximately one in every 600 dogs. The average age encountered was 10 years. There was no sex predisposition or a definite breed predisposition evident in this material. The numerical increase of canine pulmonary tumors in recent years was not explained with certainty, but may be attributed to better diagnostic methods.

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SUMMARIO IN INTERLINGUA

Tumores Pulmonar Primari in le Can: Reporto de Dece-Sex Casos

Le scrutinio del litteratura resultava in un collection de 103 descriptiones de canin tumores pulmonar primari. Sexanta-tres de illos esseva classificate como adenocarcinomas. In le presente reporto, 16 casos de primari carcinoma pulmonar es addite. Illos esseva diagnosticate in le curso de 9.282 necropsias de canes effectuate durante le periodo de 31 annos ab le 1 de julio 1928 usque al 1 de julio 1959. Super le base de lor structura histologic e le sito de lor origine, le tumores esseva classificate como adenocarcinomas bronchiolar, carcinomas bronchial, e adenomas bronchial.

Le adenocarcinomas bronchiolar del can pareva haber lor origine focal primarimente in le peripheria del pulmon. Histologicamente illos exhibiva un sol rango de cellulas cuboide in disposition papillar super tenue septos de tissu conjunctive.

Esseva trovate que le carcinomas bronchial habeva lor origine in micre bronchos secundari e tertiari in que le mucosa esseva characterisate per hyperplasia e metaplasia squamose. Duo del carcinomas bronchial habeva un transition squamose, durante que le tertie esseva del typo de micre cellulas non-differentiate. Omne le tres carcinomas bronchial habeva producite metastases, durante que solmente duo del 12 adenocarcinomas bronchiolar monstrava invasion lymphatic e metastase de nodo bronchial. Le adenocarcinomas bronchiolar constitueva tres quartos de omne le primari neoplasmas pulmonar studiate.

Le incidentia de neoplasma pulmonar esseva approximativemente un in 600 necropsias canin. Le etate medie del animales afficite esseva 10 annos. Esseva incontrate in iste material nulle predisposition per sexo e nulle definite predisposition per racia. Le augmento numeric del constatationes de canin tumores pulmonar in recente annos non esseva explicate con certitude, sed illo es possibilemente attribuibile al meliorate methodos diagnostic.

Neuropathology of Canine Toxoplasmosis

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THE DISCOVERY of *Toxoplasma gondii* as one of the causative agents of neonatal encephalomyelitis in human infants stimulated active research in this field. The improvement of laboratory techniques facilitated the diagnosis of toxoplasmosis and added to the recognition of this disease as an important congenital infection in man. Research workers in veterinary medicine investigated toxoplasmosis in wild and domestic animals, isolated and identified the organism, and succeeded in reproducing the disease experimentally.

Numerous reports on canine toxoplasmosis have appeared in the literature in recent years. The percentage of animals affected with toxoplasmosis is higher than indicated by clinical and postmortem diagnoses. Serologic examinations have shown a frequent occurrence of latent toxoplasmosis in dogs. We reported positive dye-test titers in 8 per cent of 477 dogs from Ohio.[6] Feldman[8] reported positive titers in as many as 50 per cent of the dogs in Pennsylvania and 86 per cent of the dogs in Honduras. Lainson[15] found that 48 of 113 serum samples (42.5%) from dogs in London reacted to the complement-fixation test for toxoplasma at a dilution of 1:8.

Hartley[12] found toxoplasmosis to be the third most common cause of death in 50 young pups examined in a laboratory in New Zealand.

A large proportion of dogs afflicted with toxoplasmosis developed neurologic signs. Fankhauser[7] observed clinical signs of meningoencephalitis in 8 of 11 spontaneously infected dogs. He described the signs in detail and stated that they were indistinguishable from those observed in encephalitis caused by agents other than *T. gondii*. He also described the lesions of the central nervous system (CNS)[7] and emphasized their vascular spread.[9] Except for occasional submeningeal hemorrhage and hyperemia, no gross lesions of the brain were discernible. The microscopic examination of the brain and meninges revealed perivascular infiltration of vascular as well as intramural cell elements, diffuse and focal infiltration of the meninges by lymphocytes, plasma cells and histiocytes, and parenchymal foci of glial proliferation. The glial foci sometimes had necrobiotic processes. Hemorrhage, edema, necrosis, and neuronal degeneration were frequently found. *Toxoplasma gondii*, found extracellularly and in cysts, confirmed the diagnosis.

Several workers from many countries have reported lesions of the CNS in canine toxoplasmosis.[2-5, 7, 11, 18-20] Cohrs[2] found brain lesions as early as six days after intraperitoneal injection of *T. gondii* into young pups. The lesions were described as focal necroses and granulomas. No definite explanation of the pathogenesis of these lesions was given. It was suggested that variation in virulence of the strains might be responsible for the production of either necrosis or granulomas, as is the case in some bacterial infections. This idea was abandoned since both processes occasionally occurred when a single strain was used for all experimental animals. The variation in host resistance was then considered, but this possibility was excluded when necrosis as well as glial granulomas were found in the brain of the same animal. The final conclusion was that necrosis is produced by large numbers of rapidly multiplying organisms. Where smaller numbers of organisms are present, a more proliferative granulomatous reaction is to be expected. Our experiments led to a different conclusion, as will be discussed later.

Jacobs *et al.*[13] stressed the high susceptibility of young dogs to experimental toxoplasma infection. Campbell *et al.*[1] reported concurrent distemper-toxoplasma infection. Chamberlain *et al.*[2] produced congenital toxoplasmosis in offspring by inoculation of the dam.

The objectives of this investigation were (1) to study the central nervous systems of a large number of dogs with experimentally induced or confirmed, naturally occurring toxoplasmosis; (2) to describe and classify the brain lesions and determine their type and nature in different stages of the disease; (3) to investigate the susceptibility of dogs of various ages to cerebral toxoplasmosis; and (4) to determine the distribution of the lesions in various areas of the brain and spinal cord.

MATERIALS AND METHODS

The brains and spinal cords used for this investigation are in the collection of the Department of Veterinary Pathology at the Ohio State University.

Sixty-three dogs with confirmed toxoplasmosis were studied; 43 of the infections were experimental and 20 occurred naturally. Ten pups were offspring of infected dams. They had signs of illness at birth or shortly thereafter and were classi-

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fied as having congenital infections. With 2 exceptions, the dogs studied in this investigation were either inoculated with virulent organisms (experimental cases) *T. gondii* was isolated from their organs at necropsy. Two dogs were included on the basis of their cytoplasm-modifying titers (dye test according to Sabin-Feldman) of 1:64 and 1:256, respectively. Isolation, as used in this report, refers to the mouse inoculation technique previously described.[8] The expression "demonstration" indicates the recognition of extracellular, intracellular, and encysted forms of *T. gondii* in tissue sections stained with the hematoxylin-eosin technique.

Most of the experimentally infected dogs were inoculated with approximately 1.6×10^6 *T. gondii* of the RH strain propagated in the peritoneal cavity of mice. A few dogs were inoculated with pooled organs of infected mice. The routes of inoculation were: oral, 5 dogs; subcutaneous, 11 dogs; intratracheal, 8 dogs; conjunctival sac, 3 dogs; intraocular, 2 dogs; intraperitoneal, 2 dogs; and intravenous, 7 dogs. The ages of the dogs ranged from newborn to 4 years. All clinical and laboratory data, including ages of the dogs and incidence and distribution of the lesions in the CNS, are summarized and listed in tables 1 to 3.

The brains were fixed in 10 per cent isotonic neutral formalin. Sections were taken from the spinal cord, medulla (posterior and anterior to the obex), pons, cerebellum, hypothalamus, basal ganglia, thalamus, and cerebral cortex. All sections were embedded in paraffin, cut at 6 μ, and stained with hematoxylin and eosin. The following histochemical or staining techniques were employed: luxol-fast blue, periodic acid-Schiff, hematoxylin[17]; Von Kossa's technique for phosphates; toluidine blue for Nissl bodies: and Weil-Weigert's myelin stain.

RESULTS

The large number of toxoplasma-infected dogs in this series permits presentation of the results in a manner which will give the sequence of pathologic changes as they occur in the CNS.

EUTHANASIA OR DEATH WITHIN TEN DAYS AFTER INFECTION

Fourteen experimentally infected dogs and 1 naturally infected dog were included in this group. All animals with severe illness had fever, depression, diarrhea, and respiratory distress. Two dogs aborted, 1 had convulsions, and 1 had ataxia. One dog was euthanatized *in extremis;* all others died in the acute stage. One animal was 8 months old, and all others were 5 months of age or younger (table 3).

TABLE 3—Experimental and Naturally Occurring Canine Toxoplasmosis

Dog. No.	Infection	History	Age*	Titer**	*T. gondii* Isol.	*T. gondii* Histol.	Central nervous system
1 (51542)	E	Died p.i.d. 4; severe illness.	4 mo.	.....	pos.	pos.	Perivascular edema.
2 (A446)	E	Killed p.i.d. 4; *in extremis.*	8 mo.	.....	.....	pos.	Perivascular edema.
3 (51638)	E	Died p.i.d. 5; after depression, abortion, and convulsions.	.....	.....	pos.	pos.	Perivascular edema.
4 (51530)	E	Died p.i.d. 5; severe illness.	.....	.....	pos.	pos.	Edema, focal necrosis.
5 (5224)	E	Died p.i.d. 5; abortion, severe illness.	.....	.....	pos.	.....	Nonspecific changes.
6 (551179)	E	Died p.i.d. 5; severe illness.	3 mo.	.....	pos.	pos.	Focal necrosis, free *T. gondii*, small *cysts.*
7 (551189)	E	Died p.i.d. 6; severe illness.	3 mo.	.....	pos.	pos.	Focal necrosis, free *T. gondii*, small *cysts.*
8 (551190)	E	Died p.i.d. 6; severe illness.	3 mo.	.....	pos.	pos.	Focal necrosis, free and intracellular *T. gondii, cysts.*
9 (A1699)	E	Died p.i.d. 6; severe illness.	3 mo.	.....	pos.	pos.	Focal necrosis, degeneration of vascular walls, edema, free *T. gondii.*
10 (551191)	E	Died p.i.d. 7; severe illness.	3 mo.	.....	pos.	pos.	Focal necrosis, free *T. gondii*, early glial nodules.
11 (551331)	E	Died p.i.d. 7; severe illness.	5 mo.	.....	pos.	pos.	Focal necrosis, free *T. gondii*, and *cysts.*
12 (551332)	E	Died p.i.d. 7; severe illness.	4 mo.	.....	pos.	pos.	Focal necrosis, free *T. gondii*, and *cysts.*

TABLE 3 (continued)—Experimental and Naturally Occurring Canine Toxoplasmosis

Dog. No.	Infection	History	Age*	Titer**	*T. gondii* Isol.	*T. gondii* Histol.	Central nervous system
13 (A225)	E	Died p.i.d. 7; severe illness.	5 mo.	.....	pos.	pos.	Slight perivascular and intramural perithelial cell proliferation.
14 (551337)	E	Died p.i.d. 8; severe illness.	4 mo.	.....	pos.	pos.	Focal necrosis, free and intracellular *T. gondii*.
15 (51428)	N	Died during acute illness; ataxic.	3½ mo.	1:64	pos.	pos.	Focal necrosis, marked glial proliferation, free *T. gondii*.
16 (B328)	E	Died p.i.d. 12; severe illness.	4 mo.	.....	pos.	pos.	Perithelial cell proliferation, early glial nodules, focal necrosis, free *T. gondii*.
17 (551345)	E	Died p.i.d. 13; severe illness.	6 mo.	.....	pos.	pos.	Early glial nodules.
18 (B327)	E	Died p.i.d. 16; fever, depression, tremors, ataxia.	3 mo.	.....	pos.	pos.	Edema, neuronal degeneration, necrosis, free *T. gondii*, and *cysts*.
19 (551365)	E	Killed p.i.d. 26; mild illness.	5 mo.	1:16	neg.	neg.	Negative.
20 (A350)	E	Killed p.i.d. 27; tonsillitis, recovery.	3 yr.	1:16	neg.	pos., brain only	A few cuffings, a single glial nodule with a few *T. gondii*.
21 (50976)	E	Killed p.i.d. 29; mild illness, recovery.	1½ yr.	1:256	pos.	pos.	Perivascular cuffings of microglia, lymphocytes, plasma cells.
22 (50880)	E	Killed p.i.d. 30; mild illness, recovery.	1½ yr.	1:16	neg.	neg.	Negative.
23 (551367)	E	Died p.i.d. 31; from nephritis.	4 mo.	1:16	neg.	pos.	Negative.
24 (551370)	E	Killed p.i.d. 33; mild illness, recovery.	2 yr.	1:256	pos.	neg.	Perivascular cuffings, glial scars.
25 (A224)	E	Killed p.i.d. 34; tonsillitis, recovery.	11 mo.	1:64	neg.	pos.	Glial nodules.
26 (51357)	E	Killed p.i.d. 35; progressing illness.	2½ mo.	1:128	pos.	pos.	Negative.
27 (50882)	E	Killed p.i.d. 37; progressing illness.	1½ yr.	1:128	pos.	pos.	Negative.
28 (51358)	E	Killed p.i.d. 37; progressing illness.	2½ mo.	1:16	pos.	pos.	Focal necrosis, glial nodules.
29 (551366)	E	Killed p.i.d. 45; moderate illness, recovery.	4 mo.	1:64	pos.	pos.	Cuffings, glial nodules, *cysts*.
30 (551369)	E	Killed p.i.d. 46; mild illness, recovery.	8 mo.	neg.	neg.	neg.	Slight perivascular cuffings.
31 (51607)	E	Killed p.i.d. 60; asymptomatic.	adult	1:4,096	neg.	neg.	Perivascular cuffings.
32 (551368)	E	Killed p.i.d. 82; mild illness, recovery.	8 mo.	1:64	neg.	neg.	Negative.
33 (56618)	E	Killed p.i.d. 116; endophthalmitis following intraocular inoculation.	3 yr.	1:16	neg.	neg.	Glial nodules.
34 (50879)	E	Killed p.i.d. 121; complete recovery.	2 yr.	1:2,560	neg.	neg.	Cuffings, glial nodules.
35 (56616)	E	Killed p.i.d. 123; endophthalmitis following intraocular inoculation.	2½ yr.	1:64	neg.	neg.	Necrosis.
36 (56183)	E	Killed p.i.d. 145; asymptomatic.	3 yr.	1:16	neg.	neg.	Negative.
37 (51227)	E	Killed p.i.d. 607; mild illness, recovery.	2 yr.	1:1,024	.....	.....	Negative.
38 (51639)	E	Killed p.i.d. 785; asymptomatic.	6 mo.	1:8	.....	.....	Perivascular cuffings, glial nodules.
39 (50877)	E	Killed p.i.d. 1,176; complete recovery.	1 yr.	1:256	neg.	neg.	Perivascular gliosis.
40 (C965)	N	Respiratory signs, paralysis.	6 mo.	.....	.....	pos., brain	Glial nodules, necrosis, demyelination, *cysts*, subependymal lesions.

TABLE 3 (continued)—Experimental and Naturally Occurring Canine Toxoplasmosis

Dog. No.	Infection	History	Age*	Titer**	*T. gondii* Isol.	*T. gondii* Histol.	Central nervous system
41 (5124)	N	Emaciation, dehydration, anorexia.	2 yr.	1:64	neg.	neg.	Focal and perivascular microglial proliferation.
42 (51509)	N	Conjunctivitis, tonsillitis.	4 yr.	1:64	pos.	pos.	Nonspecific change.
43 (51526)	N	Respiratory and nervous signs in 12 of 27 dogs in kennel.	11 mo.	1:256	pos.	pos.	Focal and perviascular microglial proliferation.
44 (5225)	N	Incoordination, posterior weakness.	3 yr.	1:64	pos.	pos.	Focal necrosis and glial proliferation.
45 (5286)	N	Pyrexia, clonic spasms, incoordination.	5 mo.	1:32	pos.	neg.	Negative.
46 (52160)	N	Conjunctivitis, chorea, depression.	6 mo.	neg.	neg.	pos.	Cuffings, glial nodules, *T. gondii*.
47 (541203)	N	Fever, depression, pneumonia, diarrhea.	5 mo.	.....	.....	pos.	Focal necrosis and glial proliferation.
48 (561732)	N	Convulsive seizures, fever, nasal discharge.	1 yr.	.....	.....	pos.	Infarcts, necrosis, free *T. gondii*, *cysts*.
49 (50278)	N	Coincidence with human toxoplasmosis in same household.	3 yr.	1:64	pos.	pos.	Edema, neuronal degeneration.
50 (5136)	N	Asymptomatic.	2½ yr.	1:256	neg.	neg.	Perivascular and focal necrosis with adventitial and glial proliferation.
51 (51355)	N	Asymptomatic.	2 yr.	.....	.....	pos.	Cuffings, necrosis, glial nodules, *T. gondii*, mineralization.
52 (A1691)	N	Asymptomatic.	9 mo.	.....	.....	pos.	Cuffings of microglia and proliferated perithelial cells, necrosis.
53 (A1769)	N	Mild illness for 3 weeks, progressing to death.	3 yr.	.....	.....	pos.	Negative.
54 (C443)	N	Anoxexia, dyspnea, fever, depression.	4 mo.	.....	.....	pos.	Glial nodules, *cysts*.
55 (51827)	E (cong.)	Died; born 16 days after inoculation of dam (dog 31).	1 wk.	.....	pos.	pos.	Focal necrosis, glial nodules, free *T. gondii*, and *cysts*.
56 (51839)	E (cong.)	Died; born 16 days after inoculation of dam (dog 31).	5 days	.....	pos.	pos.	Focal necrosis, glial nodules, free *T. gondii*, *cysts*.
57 (51840)	E (cong.)	Died; born 16 days after inoculation of dam (dog 31).	1 wk.	.....	pos.	pos.	Focal necrosis, glial nodules, free *T. gondii*, *cysts*.
58 (5240)	E (cong.)	Aborted, p.i.d. 4; from bitch No. 5.	Aborted fetus	.....	pos.	neg.	Negative.
59 (53780)	E (cong.)	Dam serologically positive.	.....	neg.	neg.	pos.	Focal necrosis and microglial proliferation.
60 (A1420)	N (cong.)	Whole litter of 9 pups affected, 5 already dead. Signs: diarrhea,dyspnea, ataxia.	6 wk.	.....	.....	pos.	Glial nodules, necrosis, free *T. gondii*, *cysts*, ruptured *cysts*.
61 (A1421)	N (cong.)	From same litter as No. 60.	6 wk.	.....	.....	pos.	Glial nodules, necrosis, free *T. gondii*, *cysts* and ruptured *cysts*.
62 (C758)	N (cong.)	Second litter from same bitch as No. 60 and 61; ataxia, paresis since birth.	8 wk.	neg.	neg.	pos. brain	Glial nodules, intact and ruptured *cysts*.
63 (C857)	N (cong.)	Same litter as No. 62	8 wk.	.....	.....	pos. brain	Glial nodules, *cysts*.

E = experimental infection; N = naturally occurring infection; cong. = congenital infection; Isol. = isolation of organisms; Histol. = histologic demonstration in one or more organs; p.i.d. = postinoculation day.
* Age of all experimental dogs refers to age at the time of inoculation. ** Cytoplasm-modifying test.

Histopathology of the Central Nervous System

Except in 1 pup, there were no parenchymatous lesions or organisms in the CNS of 5 dogs which died as early as the fourth and fifth day after inoculation. The only lesions detected were confined to small vessels and consisted of endothelial and perithelial swelling and, occasionally, necrobiotic processes and proliferation of perithelial cells. The Virchow-Robin spaces were dilated and usually contained strands of a proteinic material. These findings indicated early damage to the vascular walls, increased vascular permeability, perivascular edema, and reactive proliferation of adventitial cells.

The 9 dogs of this group which died after the fifth postinoculation day (p.i.d.) and 1 of 4 which died on p.i.d. 5 had more specific lesions and *T. gondii* in sections of the CNS. The capillaries, precapillaries, arterioles, and venules had changes similar to, but more severe than, those described in the preceding paragraph. Necrobiotic processes, manifested by pyknosis, karyorrhexis, and karyolysis, as well as perithelial attempts at repair, were obvious. Monocytes, desquamated endothelial cells, lymphocytes, and a few neutrophils lined or completely filled the lumina of the vessels. Extracellular round or oval *T. gondii* were frequently seen scattered throughout the necrotic vascular walls or in groups in the dilated perivascular spaces (fig. 1). Organisms also invaded the brain parenchyma and appeared to be in a stage of uninhibited multiplication. The parasitic invasion was accompanied by tissue necrosis (fig. 2). The cellular reaction was minimal in the beginning and consisted mainly of proliferation of microglia. Local astrocytes, oligodendroglia, and ganglion cells showed degenerative changes. Although the free forms of Toxoplasma prevailed in these acute infections, intracellular forms and small cysts (pseudocysts) with 4 to 8 organisms were seen as early as p.i.d. 5. The most striking lesions in all dogs of this group were the foci of coagulation necrosis. Their sizes ranged between 0.1 and 0.3 mm. Their relationship to vessels with diseased walls was usually demonstrated. The foci of necrosis appeared to represent extensions of a process which started in the vascular wall, and their unipolar distribution clearly distinguished them from perivascular cuffings in viral diseases.

The cellular reaction gradually increased from p.i.d. 5 to 8. Pleomorphic microglia appeared in increasing number within and at the margin of the necrotic foci and loosely filled the defect. Local astrocytes and oligodendroglia showed activation or regression, as evidenced by karyorrhexis and karyolysis. A few perithelial cells, recognized by their elongated

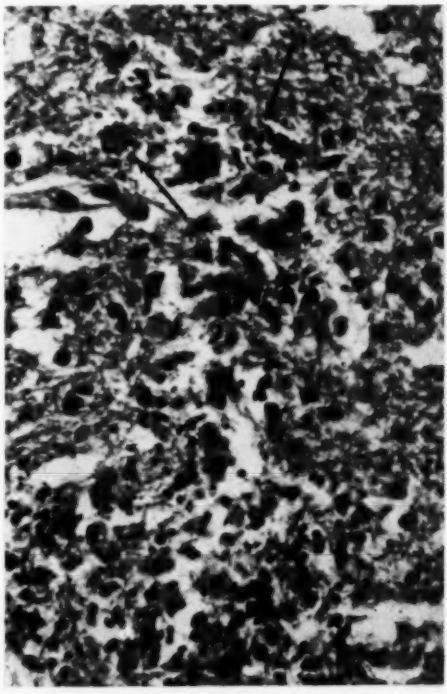

Fig. 2—Extensive necrosis and numerous extracellular proliferating Toxoplasma gondii (arrows) in the cerebellum of dog 8, six days after inoculation. H & E stain; x 625.

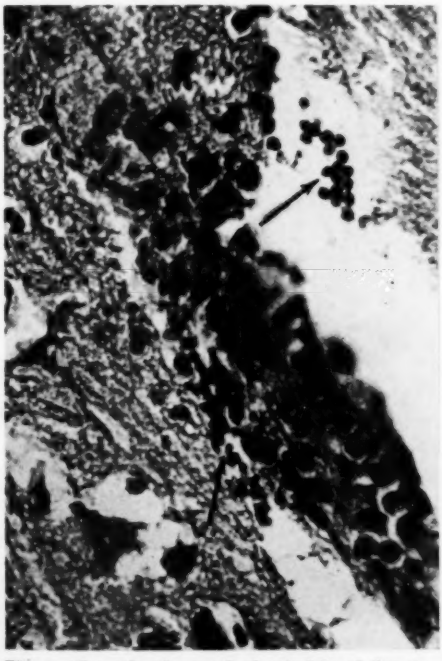

Fig. 1—Necrosis of a cerebral arteriole of dog 8 on the sixth postinoculation day. Notice the group of Toxoplasma gondii in the distended Virchow-Robin space (arrow). H & E stain; x 680.

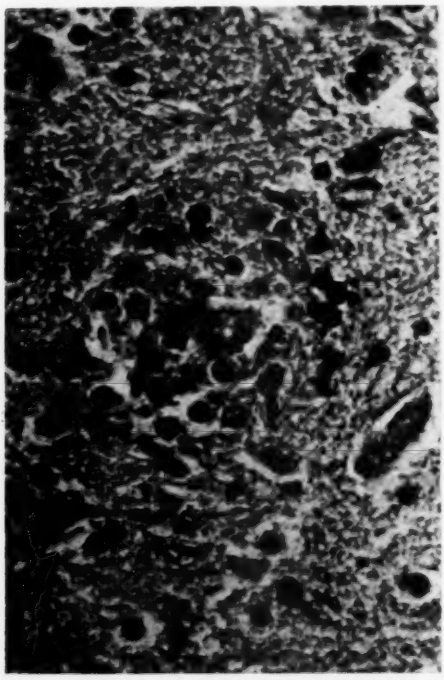

Fig. 3—Early glial nodule composed mainly of pleomorphic microglia. Central necrosis and nuclear fragmentation. (dog 10, died postinoculation day 7). H & E stain; x 760.

or handle-shaped nuclei, and one or two monocytes also infiltrated these areas.

By p.i.d. 7 to 8, these cells had formed an early glial nodule (fig. 3). These nodules were characteristic of the more advanced cases in this group and represented a transition to the changes found in dogs which died more than ten days after infection. The lesions of the vascular walls, being in a process of repair, still had evidence of necrosis at this stage. In the meninges, there was only slight infiltration of lymphocytes and plasma cells.

EUTHANASIA OR DEATH AFTER THE TENTH DAY OF INFECTION

In this group of dogs there were 24 experimental and 15 naturally occurring cases. While the dogs in the previous group, with 1 exception, did not exceed the age of 5 months, the dogs in this group were 3 months to 4 years old. Since the dogs of this group had distinctly different clinical patterns, they were subclassified under two headings; (1) subacute and chronic symptomatic toxoplasmosis, and (2) latent toxoplasmosis.

Subacute and Chronic Symptomatic Toxoplasmosis.—All dogs of this group either died from toxoplasmosis or were ill at the time of euthanasia, performed in the absence of another disease. Nine dogs were experimentally infected; 11 were naturally infected. Eight of the 11 naturally infected dogs had nervous signs consisting of hyperexcitability, depression, tremors, clonic spasms, head tilt, incoordination, ataxia, posterior weakness, paralysis, and convulsive disorders.

Histopathology of the Central Nervous System.—The early glial nodule, as described for the dogs of the previous group, still was the most impressive lesion in dogs 16, 17, and 18. These dogs had died on p.i.d. 12, 13, and 16, respectively. The glial nodules showed increased cellularity, less necrosis, and a decreased number of or-

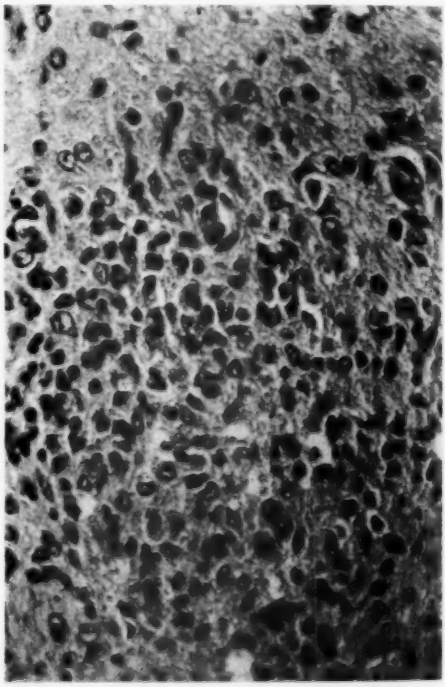

Fig. 4—Well-developed glial nodule in cerebral cortex, composed of macro- and microglial cell elements and mesenchymal cells from the perivascular adventitia. From dog 63, with a naturally occurring congenital case. H & E stain; x 475.

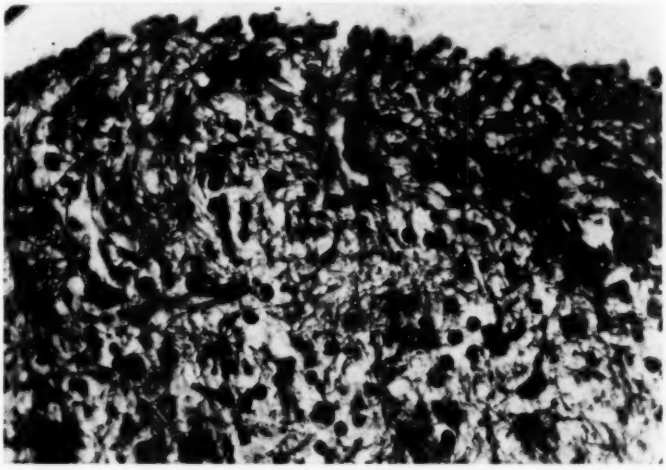

Fig. 5—Periventricular scar in medulla of dog 24, 33 days postinoculation. The vessels are pronounced and show adventitial cell proliferation and perivascular fibrosis. H & E stain; x 380.

ganisms which only occasionally were seen as free extracellular forms. They were usually found in cysts located at the margins of the lesions. In the dogs killed four to six weeks after inoculation, the lesions in the CNS appeared to be in an advanced process of healing. The glial nodule completely filled the parenchymal defect and only occasional pyknotic or fragmented nuclei indicated necrobiosis. The nodules were clearly demarcated and ranged from 120 to 300 μ in diameter. In cases of severe involvement of the CNS, several nodules coalesced and formed a rather diffuse lesion, sometimes extending over areas up to 0.5 x 0.5 cm. The isolated glial nodule (fig. 4) had a characteristic pattern. Approximately two thirds of the cells were microglia, pleomorphic as well as rod-cell types. There was no typical distribution or arrangement of these two microglial cell types. Astrocytes and oligodendroglia varied in number in the individual dog or nodule. The astrocytic nuclei within and in close vicinity to nodules appeared to be larger and more vesiculated than normal. The oligodendroglial nuclei were hypochromatic, suggesting activation. Gemistocytes (plump astrocytes, gemästete glia) were only occasionally observed. Perithelial cells usually participated in the formation of glial nodules, although the relationship to a diseased vessel could not always be traced. One or two monocytes with indented nuclei were sometimes seen and could have come either from the bloodstream or the adventitia. The presence of vascular cell elements, such as lymphocytes or neutrophils, in glial nodules was extremely rare. The lesions in the vascular walls had increased cellularity and decreased necrosis as the disease progressed. Some vessels had prominent cuffings, consisting mainly of proliferated perithelial cells. A month after inoculation, plasma cells appeared in the perivascular infiltrates and were sometimes, as in dog 29, the cells most frequently observed. In more chronic cases, the perivascular reaction was based mainly on adventitial cell proliferation and production of a fibrous material, suggesting advanced repair and scarring. This stage was already reached at an earlier date in animals which had only mild, short illnesses and recovered within two weeks. For example, dog 24, destroyed 33 days after inoculation in a stage of clinical health, had prominent vascular centers of organization in a periventricular area of vacuolization (fig. 5). Periventricular lesions, as such, are extremely rare in animals and in only two instances have we found lesions associated with involvement of the ependyma (fig. 6). In all other cases, the ependyma was intact and foci of necrosis or glial nodules in the vicinity of ventricles always originated from damaged vessels as in other parts of the CNS. *Toxoplasma gondii* was not demonstrated in the CNS of experimentally infected dogs killed after p.i.d. 45. In dogs with naturally occurring chronic

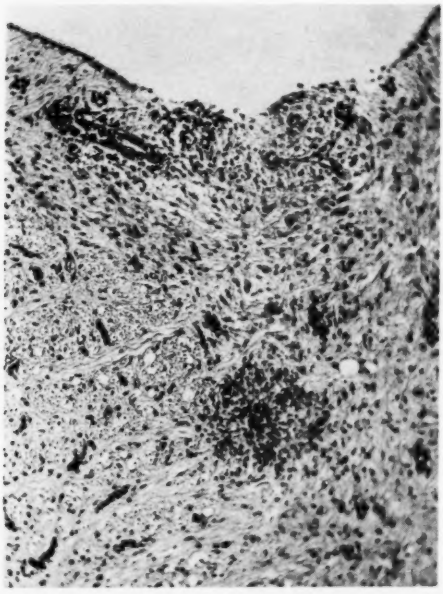

Fig. 6—Subependymal lesion in a dog with naturally occurring chronic toxoplasmosis (dog 40). Notice the absence of ependymal cells in this area and the diffuse and perivascular cellular infiltration. H & E stain; x 100.

cases where the lesions were more extensive, Toxoplasma cysts were usually observed either in the vicinity of glial nodules and vascular lesions, or in locations remote from any reaction (fig. 7, 8). Several naturally infected dogs (40* and 51 and dogs 60–62, with congenital toxoplasmosis) had healing lesions, indicated by glial nodules and scarring, in some parts of the CNS and highly reactive lesions in other areas. The active lesions were characterized by necrosis and free *T. gondii*. In these dogs, ruptured cysts (fig. 9, 10) were frequently found which suggested a reactivated chronic or latent process. The extensive areas of demyelination with numerous gitter cells (fig. 11) pointed to the possibility of a hyperergic response. The prominent perithelial cell proliferation was attributed to Toxoplasma or fatty acids released by demyelination, or both. Infarcts with resulting ischemia were observed in 1 dog (48). This 1-year-old dog had convulsive seizures for four days, which became progressively worse until they occurred every 15 minutes. A constant elevated temperature of 103 F. was recorded. Euthanasia was performed after the dog became semicomatose on the fourth day. Interstitial pneumonia, congestion of the liver, healed infarcts of the kidney, and two recent hemorrhagic infarcts in the brain (fig. 12) were found at necropsy. Both infarcts measured approximately 0.5 cm. in diameter and were located in the right ectosylvian gyrus and in the left anterior sylvian gyrus respectively. Numerous cysts and free organisms were seen in all sections of the lung. Focal necrosis (but no organisms) was found in the liver.

Microscopic examination of various parts of the brain revealed focal necrosis and gliosis, marked edema, and nonsuppurative meningitis. The meningitis was most severe in sections taken from the areas over the infarcts (fig. 13). The infiltrating cells were plasma cells, lymphocytes, mononuclear cells, and large, elongated, hypochromatic cells resembling fibroblasts. The meningeal vessels were severely injured.

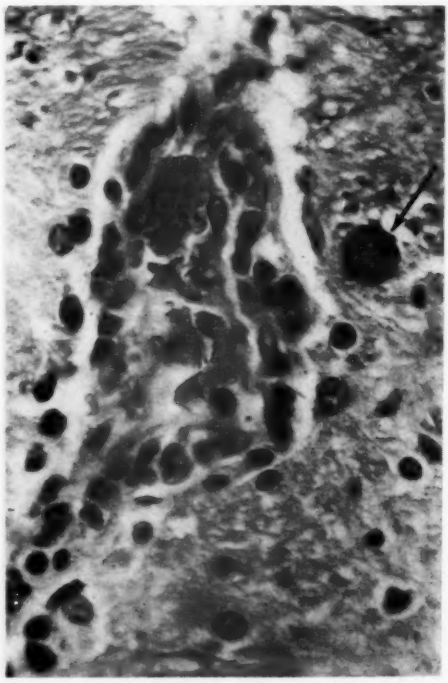

Fig. 7—A Toxoplasma cyst (arrow) adjacent to a lesion involving an arteriole in dog 64. H & E stain; x 760.

* Made available through the courtesy of Dr. George Sayre, Mayo Clinic, Rochester, Minn.

Fig. 8—A Toxoplasma cyst remote from any reaction; dog 64. H & E stain; x 1,500.

The arterial intima was rough and projected into the lumen with several irregular apices. The muscularis appeared hypertrophic. Smaller arteries and veins had regressive changes or destruction of their walls. The walls of many arteries were thickened and a large amount of disorganized material, positive to periodic acid-Schiff (PAS), was deposited in the perivascular areas. The perivascular areas, as well as the meninges, contained numerous Toxoplasma cysts. The cyst walls were distinct and showed a positive PAS reaction. Numerous large cells contained proliferating Toxoplasma in their cytoplasm. These large cells had pyknotic nuclei in an eccentric position, which were apparently displaced by organisms. There was marked infiltration of cells which could not be identified due to advanced nuclear degeneration. Large areas of hemorrhage and ischemic necrosis of the cortex extended deep into the tissue. The vessels within the necrotic fields resembled those of the meninges. The walls of vessels were either thickened or consisted of thin irregular fragments. A few degenerated, oval, hypochromatic cells indicated remnants of the vessel wall (fig. 14). Vessels from the periphery of the necrotic area were less damaged but had pronounced perivascular infiltration of plasma cells, lymphocytes, microglia, monocytes, and proliferating perithelial cells. The same cell types were present in the perinecrotic zone. Plump degenerated astrocytes, pleomorphic microglia, and rod-cells were also observed. At the margin of the lesion there were numerous cysts, ruptured cysts, and extracellular, proliferating organisms. An extensive area of demyelination with a large number of gitter cells was present. The ganglion cells within the lesion were in various stages of neuronal degeneration.

Latent Toxoplasmosis.—This group consisted of 15 experimentaly infected and 4 naturally infected dogs. Of the 15 experimentally infected dogs, 3 remained clinically healthy and 12 recovered from mild or moderate illnesses. The dogs were killed one to four months after inoculation. *Toxoplasma gondii* was demonstrated in the

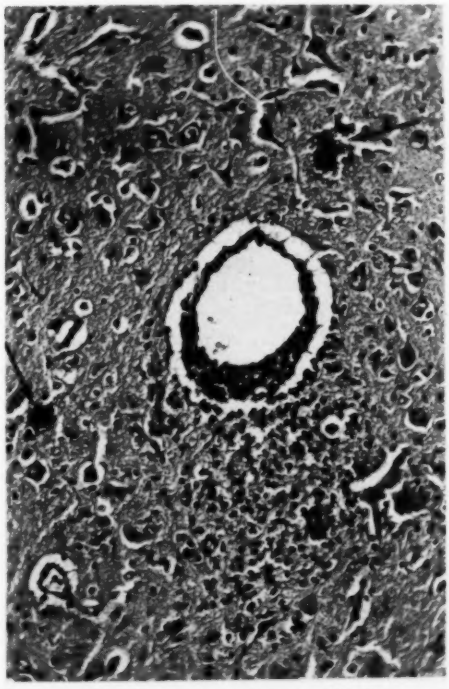

Fig. 9—Rupturing cysts (arrows) indicating a reactivated process. Dog 64. H & E stain; x 165.

Fig. 10—Rupturing cyst and numerous released Toxoplasma gondii. Dog 64. H & E stain; x 1,500.

brains of 2 animals. Lesions compatible with those seen in chronic symptomatic toxoplasmosis were found in the CNS of 10 dogs. Five had no lesions. The 4 naturally infected dogs were individually different and therefore are described separately.

Dog 49 was a clinically healthy 3-year-old Coonhound which came from a household where toxoplasmosis had affected members of the family. Serum from this dog had a cytoplasm-modifying titer of 1:64. Isolation and demonstration of *T. gondii* were successful. Examination of the brain revealed mild microglial proliferation, focal necrosis, and calcified plaques which could not be related with certainty to injury by Toxoplasma.

Dog 50, 2½ years of age and clinically healthy, had an antibody titer of 1:256 in a serologic survey. Organisms neither could be isolated nor identified in tissue sections. Perivascular proliferation of perithelial cells and focal accumulation of microglia were similar to those seen in dogs with confirmed toxoplasmosis.

Dog 51, 2 years old, was admitted to the clinic with a history of coxofemoral luxation. Euthanasia was performed at the owner's request. *Toxoplasma gondii* and characteristic lesions were observed in many organs, including the CNS.

Dog 52, 9 months old, died after the administration of a vermicide. *Toxoplasma gondii* were demonstrated in the liver and characteristic lesions of chronic toxoplasmosis were found in the CNS.

CONGENITAL TOXOPLASMOSIS

Five dogs were whelped (4 pups) or aborted (1 fetus) by artificially infected dams. The other 4 pups had naturally occurring infections; signs of illness were seen at birth or shortly afterwards.

Of the dogs with experimental toxoplasmosis, 1 pup (58) was aborted four days after inoculation of the dam; it had developed no lesions in the CNS. Another pup (59) was whelped by a clinically healthy

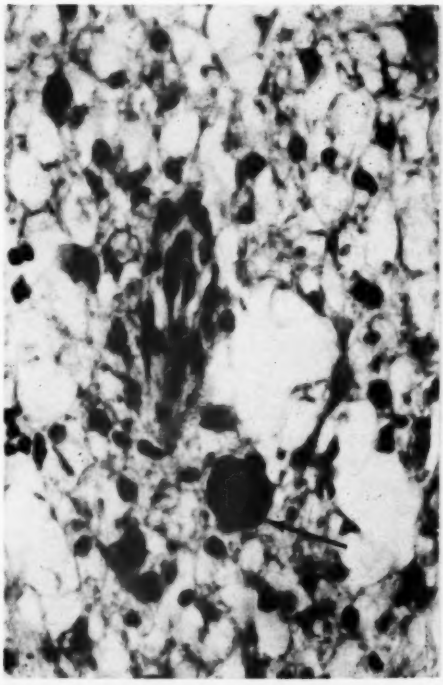

Fig. 11—A Toxoplasma cyst (arrow) in an area of demyelination. A vessel in the center shows prominent perithelial cell proliferation attributable to toxoplasma or fatty acids, or both, released by demyelination. Dog 40. H & E stain; x 540.

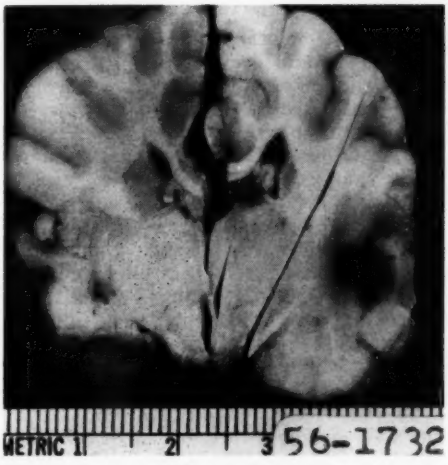

Fig. 12—Cross section through cerebrum of dog 48. Notice the infarct in the left sylvian gyrus.

but serologically positive mother. The brain sections revealed foci of necrosis and glial proliferation suggestive of a Toxoplasma infection.

The other 3 dogs with experimental infections (55, 56, 57) were littermates born 16 days after inoculation of the dam. All 3 died within the first week after parturition. *Toxoplasma gondii* was isolated from several organs of each dog. The CNS of all 3 pups had lesions similar to those observed in experimentally infected animals which died on p.i.d. 8 to 16. They appeared, however, to be more severe and extensive than in non-congenital infections. They were especially numerous in sections of the cortex. The vessels had severe lesions consisting of ballooning of endothelial cells, swelling and proliferation of perithelial cells, perivascular edema, and intramural and perivascular infiltration of lymphocytes and monocytes, even in vessels not associated with parenchymal lesions. Lesions of the CNS in apposition with vessels consisted of necrotic foci with marked glial reaction and frequently of well-circumscribed early glial nodules. *Toxoplasma gondii* was seen extracellularly in the Virchow-Robin spaces and in necrotic foci. Organisms were also found intracellularly and in cysts at the margin of lesions or remote from any reactive process.

The 4 pups with naturally occurring, congenital infections were from two consecutive litters (2 pups each) of the same bitch. All pups in both litters had diarrhea, respiratory distress, and ataxia since birth. Five of the first litter died. The 4 pups received for examination had extensive lesions of the CNS, consisting of necrosis, demyelination, glial nodules and vessels with degenerative and inflammatory changes of their walls. *Toxoplasma gondii* was demonstrated in the CNS of all 4 pups as extracellular forms as well as in cysts. Many cysts were demonstrated in a stage of rupture.

This is apparently the first report of a bitch giving birth to two consecutive litters with congenital toxoplasmosis.

DISCUSSION

The fact that toxoplasmosis is a generalized infection seems to be well established. *Toxoplasma gondii* does not show special affinity for any particular tissue or cell type. The CNS became affected, like other organs, during the stage of parasitemia which was usually reached 48 hours after infection and lasted approximately ten days. Organisms could be recovered from the bloodstream during that time. Our examinations, however, have shown that organs other than brain and spinal cord develop dramatic lesions two to three days prior to infection of the CNS.

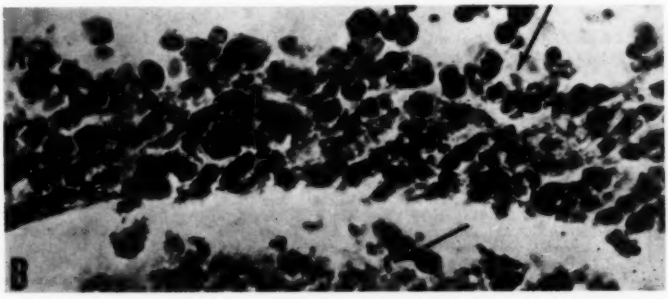

Fig. 13—Extensive leptomeningitis in dog 48. Arrows point out Toxoplasma in leptomeninx (A) and cerebral cortex (B). H & E stain; x 680.

Fig. 14—Section through infarct illustrated in figure 12. Notice diffuse ischemic necrosis, fragmentation of the vascular wall, and the perivascular deposit of disorganized PAS-positive material. Luxol-fast blue, periodic acid-Schiff, hematoxylin; x 425.

Toxoplasma gondii and specific lesions can be demonstrated in the CNS long after the visceral involvement has subsided. Specific lesions in the CNS were found in 14 dogs with chronic disease and organisms were recognized in the brains of 5 dogs, while *T. gondii* was not demonstrated in other organs. The possible role of the blood-brain barrier and its importance in the pathogenesis of cerebral toxoplasmosis will be discussed elsewhere.[14] The earliest lesions were confined to small vessels and consisted of endothelial swelling, perivascular edema, and perithelial cell proliferation. Generalized edema, extensive hemorrhage, and ischemia were not observed. *Toxoplasma gondii* appeared to actively invade the vascular walls, the Virchow-Robin spaces, and the adjacent nervous tissue by p.i.d. 5 to 6. The parasitic invasion resulted in necrosis. Free proliferating organisms were seen in large numbers in the necrotic areas. The very slight microglial reaction in the beginning gradually increased and led to the formation of the glial nodules. These nodules contained microglia activated oligodendroglia, astrocytes, infiltrating perithelial cells, and, occasionally, monocytes. They were outlined on p.i.d. 10 and well circumscribed on p.i.d. 16. Extracellular Toxoplasma were rarely found at this stage, but intracellular organisms and cysts were seen at the margin of the lesions in stages of healing and repair. Vessels appeared pronounced and functioned as centers of organization. Glial, fibrotic, or mixed scars were observed. Organisms were rarely seen at this stage and then only in cysts remote from lesions and apparently not causing any reaction. The meninges showed usually slight to moderate infiltration of lymphocytes, monocytes, and plasma cells. Plasma cells were not uncommon in perivascular infiltrates in early chronic infections and suggest an "allergic" response.

The presence of Toxoplasma cysts in the CNS of dogs which had completely recovered from the disease clinically led to the classification of latent toxoplasmosis. Nineteen animals were either clinically healthy when euthanasia was performed or died from another disease. The cytoplasm-modifying titer ranged from 1:16 to 1:4,096 in 15 of 17 animals in which the test was applied. Serum from 1 dog had a titer of 1:8 and

TABLE 1—Relationship of Age to the Occurrence of Lesions and the Presence of Toxoplasma gondii in the Central Nervous Systems of 20 Naturally Infected and 39 Experimentally Infected Dogs

	Naturally occurring infections			Experimental infections		
Age*	Lesions	*T. gondii*	Negative	Lesions	*T. gondii*	Negative
Congenital	4	4		4	3	1
1–6 mo.	5	3	1	15	11	3
6–12 mo.	2	1		2		2
Over 1 yr.	6	2	2	8	1	4
Total	17	10	3	29	15	10

Four of the experimentally infected dogs were not included in this table because their exact ages were not known.

* Age of all experimentally infected dogs refers to age at the time of inoculation.

serum from another was negative. Fourteen of the 19 dogs had characteristic lesions of toxoplasmosis in the CNS.

Reactivated toxoplasmosis was manifested histologically by rupture of cysts, necrosis, and extensive demyelination, indicating a hyperergic response. Several dogs which died or were killed in a stage of reactivation were considered to be congenitally infected. Probably most of the cases of adolescent or adult toxoplasmosis are congenital in origin and, unless reactivated, exist as latent infections for the life of the animal.

Age played an important role concerning incidence and severity of toxoplasmic lesions in the CNS (table 1). The most severe and extensive lesions were found in congenitally infected pups. Dogs which died in an acute phase of illness were, with 1 exception, less than 6 months old. Older dogs were more resistant and many did not develop clinical illness after inoculation.

The distribution of the lesions was rather uniform in various areas of the CNS; however, Toxoplasma cysts in dogs with chronic disease were more frequently found in the cerebral cortex (table 2). Subependymal lesions, although common in congenital toxoplasmosis in children, were rare in dogs. They were found in only 3 dogs and were extremely mild as compared to the extensive areas of periventricular necrosis in infants.[10] Of 63 dogs, 49 (74%) had specific lesions in the CNS. Four of the 17 negative dogs did not have extraneural lesions; 5 of the remaining 13 had peracute infections in which specific lesions of the CNS had not developed. There were only 8 dogs (10%) in which Toxoplasma infection had spread exclusively to various extraneural organs and did not affect the CNS. However, there were 14 dogs in which specific lesions appeared exclusively in the CNS.

These findings lead to the conclusion that the involvement of the CNS in canine toxoplasmosis is extremely high and that microscopic study of the CNS is of great importance in the diagnosis of toxoplasmosis in dogs.

SUMMARY

The central nervous systems (CNS) of 63 dogs with confirmed toxoplasmosis were studied; 43 had experimental infections and 20 had naturally occurring infections. Lesions in the CNS attributed to toxoplasmosis, were found in 47 dogs (74%).

TABLE 2—Distribution of Lesions and Toxoplasma gondii in the Central Nervous Systems of 47 Dogs

Location	No. of animals	
	Lesions	*T. gondii*
Spinal cord	9	4
Medulla	29	13
Pons	20	8
Cerebellum	21	9
Midbrain	28	12
Cerebral cortex and basal ganglia	47	17

Toxoplasma gondii was demonstrated microscopically in the CNS of 25 dogs. Toxoplasmosis of the CNS was characterized by vascular damage and focal necrosis in acute cases and by glial nodules and repair in chronic cases. Extracellular organisms were found in relation to necrotic foci in dogs with acute infections, and intracellular Toxoplasma and cysts were found in dogs with chronic disease. Nineteen dogs were classified as having latent infections.

Ruptured cysts and hyperergic response were characteristics of reactivated latent toxoplasmosis. Lesions were found in all parts of the CNS. Young dogs were more susceptible and congenital infections resulted in the most severe and extensive lesions. An original observation was the occurrence of congenital toxoplasmosis in two consecutive litters whelped by the same bitch.

The incidence of brain and cord lesions in canine toxoplasmosis is considered to be high and of great diagnostic significance.

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SUMMARIO IN INTERLINGUA

Neuropathologia de Toxoplasmosis Canin

Esseva studiate le systema nervose central in 63 canes con confirmate toxoplasmosis. In 43 le infection esseva experimental; in 20 illo habeva occurrite naturalmente. Lesiones del systema nervose central de un genere attribuibile a toxoplasmosis esseva incontrate in 47 canes (74%). *Toxoplasma gondii* esseva demonstrate microscopicamente in le systema nervose central de 25 canes. Toxoplasmosis del systema nervose central esseva characterisate per lesiones vascular e necrose local in casos acute e per nodulos glial e reparo in casos chronic. Organismos extracellular esseva trovate in relation a focos necrotic in canes con infectiones acute, e toxoplasma intracellular e cystes esseva trovate in canes con morbo chronic. Dece-novem canes esseva classificate como portatores de infectiones latente.

Rupturation de cystes e responsas hyperergic esseva characteristicas de reactivate toxoplasmosis latente. Lesiones esseva trovate in omne parte del systema nervose central. Juvene canes esseva plus susceptibile, e infectiones congenite resultava in le plus sever e le plus extense lesiones. Un observation original esseva le occurrentia de toxoplasmosis congenite in duo parturitiones consecutive del mesme can femina.

Es opinate que le incidentia de lesiones cerebral e del medulla dorsal in toxoplasmosis canin es alte e de grande signification diagnostic.

The Microscopic Anatomy of the External Ear of *Felis domesticus*

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SINCE RELATIVELY little histologic research has been done on the external ear of the cat, it was included in a study of the entire skin.[12] According to Speed,[11] Teger (1906) reported active sweat glands in the external auditory canal of the cat. Montagna [8] described coiled, dilated, tubular sudoriparous glands and sebaceous glands opening either into the upper portion of the hair follicle or directly onto the surface of the external auditory meatus in the external ear of the cat. Takeda [13] also studied the histologic structure of the external auditory canal of the cat and other domestic animals. Senturia and Liebmann [10] found that trauma followed by contamination with Pseudomonas organisms consistently produced pathologic changes in the external ear of the cat. Daniel and Prichard [2] described an S-shaped arteriovenous anastomosis in the ear of the cat which was less complex and smaller than that found in most animals studied.

MATERIALS AND METHODS

Twenty domestic cats (5 mature females, 3 noncastrated mature males, 1 immature female, and 1 immature male, with supplemental sections from 10 other cats) were used for this study. The animals were killed with lethal doses of sodium pentothal. Cross and longitudinal sections from the apex and scapha of the pinna; longitudinal sections of the medial and lateral walls of the meatus; and cross sections at two levels each of the upper, middle, and lower two thirds of the meatus were examined (fig. 1). The tissues were fixed in formalin-alcohol-acetic acid (Lavdowsky's mixture [4]), and embedded in Tissuemat* according to the methods of Johnson *et al.*[6] Longitudinal and frontal sections were processed with a modified hematoxylin and eosin staining technique,[7] Weigert and van Gieson's connective tissue staining process, Wright's blood stain for mast cells,[1] and Yang's [15] method for pigment determination.

A dorsolateral view of the left external ear of the cat is illustrated (fig. 1). The terminology used was patterned after that of Ellenberger-Baum.[3] Scapha foramina, previously described by Getty *et al.*[5] for the dog and illustrated by Ellenberger-Baum [3] for the horse, cow, dog, and pig are shown (fig. 2, 3).

EPIDERMIS

The external epidermis of the ear consisted of four distinct layers: stratum corneum, stratum granulosum, stratum spinosum, and stratum cylindricum. The stratum lucidum was not usually found.

Stratum Corneum.—The stratum corneum of the pinna was relatively thin and resembled that of general body areas. In the external auditory meatus, under normal conditions, the stratum corneum resembled that of the pinna, but in abnormal conditions it became hyperplastic, contributing to an altered morphology of the skin of that area (fig. 4). It increased in thickness and, in certain instances, covered the sebaceous gland openings entering the lumen of the ear canal. The general epidermal thickness increased as much as three to four times normal size. The epidermal cells appeared to migrate into the dermis and to invest the sebaceous glands.

Stratum Lucidum.—This layer was not present in the ear pinna and was not observed in the ear canal.

Stratum Granulosum.—The stratum granulosum was present and usually consisted of one to two layers, although these layers were not as conspicuous as those observed in some general skin sections.

Stratum Spinosum.—The stratum spinosum of the pinna normally consisted of two to three layers. As a result of hyperplasia, it had as many as 38 cell layers (fig. 4).

Stratum Cylindricum.—The stratum cylindricum contained no more than one layer of columnar cells in the ear canal and pinna. Occasional mitotic figures were observed.

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Based on a thesis submitted by the senior author in partial fulfillment of the requirements for the M.S. degree at Michigan State University.

* Tissuemat, produced by Fisher Scientific Company, Pittsburgh, Pa.

XUM

DERMIS

Pinna.—The pinna was composed of a central core of elastic cartilage covered on both sides by a layer of skin (fig. 5). Depending on the area, one of the auricular muscles was present in the proximal portion of the convex border (fig. 6). The cartilage-free tip of the pinna is shown (fig. 7). The dermis had dense connective tissue, characterized by an indefinite division between the fibers of the stratum papillare cutis and stratum reticulare. The skin increased in thickness from the tip of the pinna (fig. 7) to the opening of the external auditory canal (fig. 8). The hair distribution on the convex side was usually dense, resembling that of the general body areas. The concave surface had sparse, fine hair, except toward the rostral border. Occasionally, single hair follicles resembling those of the external auditory meatus were observed. The sebaceous glands of the ear pinna were short, with crowded excretory and secretory units due to the presence of the cartilaginous septum (fig. 5). Contrary to the statement of Trautmann and Fiebiger [14] that "the concave surface contains large sebaceous and small tubular glands," they were observed on both the concave and convex surfaces of the pinna in the cat.

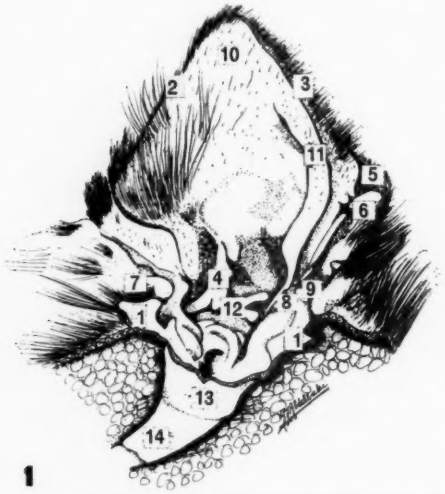
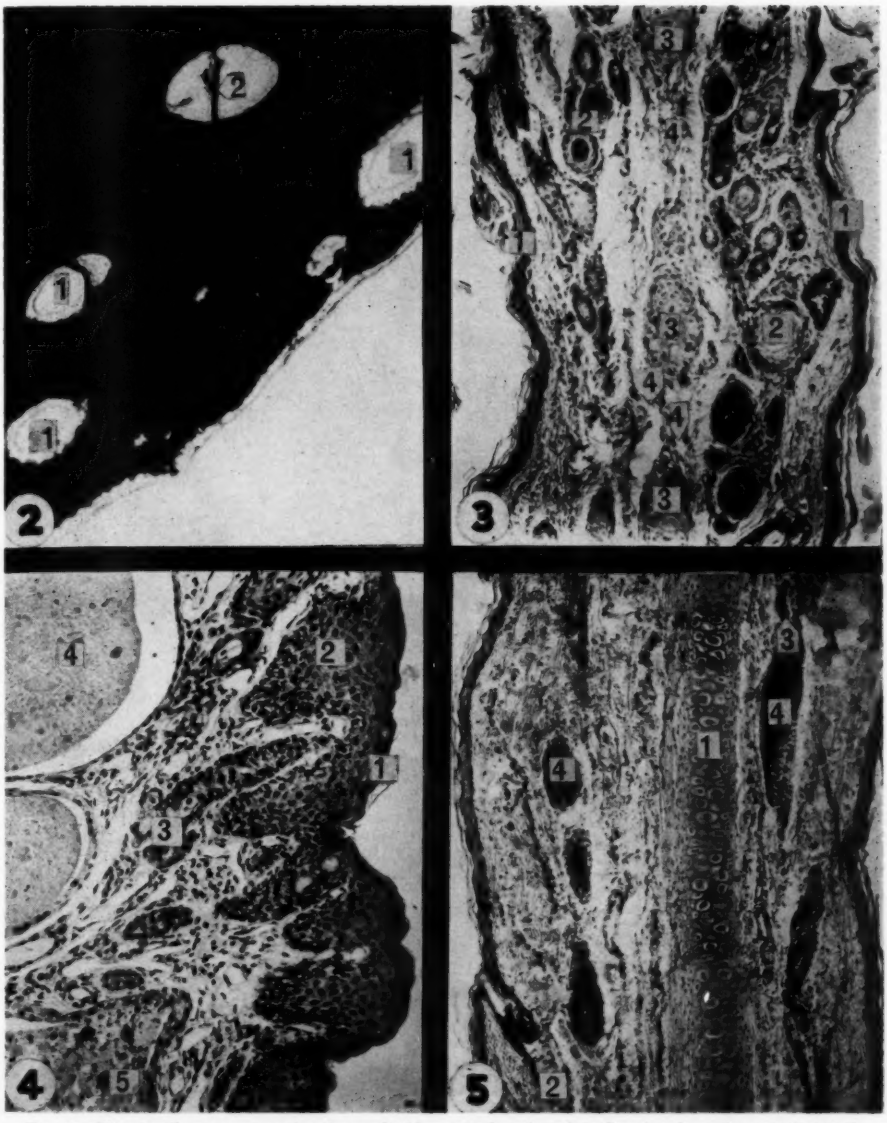


Fig. 1—Anterolateral view of the left ear of the cat. (1) Tragus incised to expose the upper third of the external auditory meatus; (2) rostral border of the pinna; (3) caudal border of the pinna; (4) tubercle of anthelix; (5) caudal auricular sulcus; (6) antitragus; (7) rostral auricular sulcus; (8) plica antitragica; (9) incisure intertragica; (10) scapha; (11) longitudinal ear folds; (12) transverse folds; (13) middle third of the external auditory meatus; and (14) lower third of the external auditory meatus.

External Auditory Meatus.—Both dermal layers were present in the external auditory canal. The fiber directions were determined by folds in the skin and by the number of sebaceous and ceruminous glands. The skin of the external auditory meatus had large and small folds in the outer and middle divisions. Small elastic and collagenous fibers surrounded the sebaceous and ceruminous glands immediately beneath the epidermis (fig. 8). This layer was referred to as the stratum papillare cutis in keeping with the size of fibers and description of skin in other body areas. In the hyperplastic specimen, a more definite papillary layer was formed (fig. 4). The fibers beneath the papillary layer were large and resembled the reticular layer in other areas, with the exception of being extremely dense. These fibers fused with the perichondrium of the elastic cartilage. The elastic fibers of the lower meatus appeared larger, more numerous, and extended parallel to the epidermis. The folded areas usually possessed a fat-laden central portion (fig. 9) which occasionally contained ceruminous glands. These folds had more surface area, resulting in less crowding of the dermal elements.

Hair was lacking or sparse in the external ear canal. When present, it usually occurred singly, associated with large sebaceous gland clusters. Trautmann and Fiebiger [14] referred to this as wool or lanugo hair. The number of sebaceous glands depended on their location in the meatus. In the outer third, in the areas with ridges and tubercles (fig. 8), there were one to two large clusters per square millimeter. In the middle meatus, this number was slightly increased, and distinctive crowding was seen in the lower third of the canal nearest the tympanic membrane. Although the largest sebaceous gland clusters in the external auditory canal measured approximately 300 μ in diameter, a variety of sizes occurred. A similar description was given by Montagna [8] for the cat and by Nielsen [9] for the dog. No mitotic figures were observed in sebaceous glands, contrary to the report by Montagna.[8]

The ceruminous glands found throughout the external meatus opened upon the sur-



Fg. 2—The cartilagenous scapha free of skin and dermis. (1) Scapha foramina; (2) blood vessel transversing a scapha foramina. x 24.

Fig. 3—Longitudinal section through the pinna with two scapha foramina showing a blood vessel in each. (1) Epidermis; (2) dermis with hair follicles and several sweat and sebaceous glands; (3) cartilaginous septum; (4) blood vessels penetrating the foramina in the septum. H & E stain; x 50.

Fig. 4—Section from lower third of the external auditory meatus showing hyperplastic epidermis, distended ceruminous glands, calcium deposits, and degenerate sebaceous glands. (1) Stratum corneum; (2) stratum spinosum; (3) degenerate sebaceous glands; (4) modified ceruminous glands; and (5) calcium deposits. H & E stain; x 100.

Fig. 5—Section of the middle portion of pinna showing the central core of elastic cartilage and dermal elements. (1) Central elastic cartilage; (2) sebaceous gland; (3) sweat gland; and, (4) hair follicles. H & E stain; x 100.

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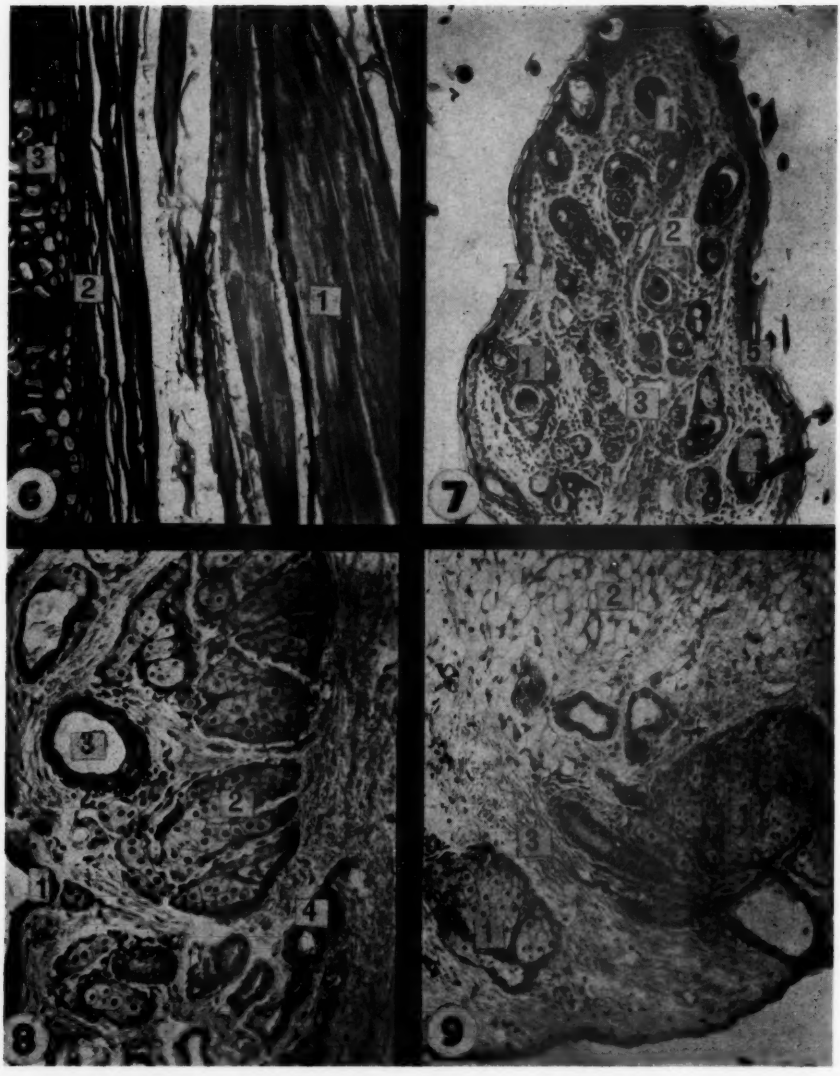

Fig. 6—Midsection of the ear pinna showing the auricular muscle. (1) Muscle; (2) perichondrium; and (3) elastic cartilage. Weigert-van Gieson stain; x 60.

Fig. 7—Tip of the pinna showing distribution of hair follicles and sebaceous and sweat glands on the convex and concave borders. (1) Hair follicles; (2) sebaceous glands; (3) sweat glands; (4) convex border; and (5) concave border. H & E stain; x 60.

Fig. 8—Section through the lower third of the external auditory meatus showing the normal condition of the area as compared with figure 4. Notice the difference in the thickness of the epidermis, sebaceous glands, and the ceruminous glands. (1) Epidermis; (2) sebaceous glands; (3) sebaceous gland ducts; and (4) ceruminous glands. H & E stain; x 60.

Fig. 9—Section of a tubercle in the anthelix showing the large less densely arranged sebaceous glands and fat-laden central portion representative of all ear folds. Compare density of sebaceous glands with figure 8. (1) Sebaceous glands; (2) adipose tissue; and (3) collagenous and elastic fibers. H & E stain; x 75.

face of the canal or into hair follicles. These glands increased in number in the lower third of the meatus. They were coiled, dilated, tubular glands similar in structure but larger than sweat glands, measuring 65 to 85 μ (fig. 8). The epithelium was tall columnar. The ceruminous glands were more numerous than the sweat glands. A similar condition in the dog was described by Nielsen.[9] Under pathologic conditions, the ceruminous glands may increase in size five to six times and become filled with cellular debris (fig. 4).

Pigment

A small amount of pigment was seen in the epidermis of the pinna and the external auditory canal. No chromatophores were observed in the dermal area.

Summary

The ear was divided into two parts: the pinna, with its central core of elastic cartilage and attached auricular muscles, and the external auditory meatus.

The epidermis of both regions was usually composed of only four layers, the stratum lucidum rarely being present. All layers were normally thin but, in hyperplastic sections, the stratum corneum and stratum spinosum increased markedly in thickness while the stratum granulosum and stratum cylindricum remained unaltered. A few mitotic figures were present in the stratum cylindricum.

The dermis of the pinna was thin and had dense connective tissue with little separation of the dermal fibers. It increased in thickness from the tip of the pinna to the external auditory canal. Both convex and concave sides contained sweat and sebaceous glands with short, crowded, excretory and secretory units. The hair distribution on the convex side was usually dense. The concave surface had sparse, fine hair, except toward the rostral border. The dermis of the external auditory meatus had both a stratum papillare cutis and a stratum reticulare, but the fiber arrangement was occasionally altered by size and number of glands present in the ear canal. The fibers of the stratum papillare cutis were small and similar to those in the hairy skin. The stratum reticulare became dense as it fused with the perichondrium of the cartilage. Elastic fibers appeared larger and more numerous in the lower third of the meatus. The number of fibers was reduced in sections showing hyperplasia.

Sebaceous glands measured from 200 to 450 μ in diameter. They sometimes occurred independently of hair follicles. Ceruminous glands were found throughout the external auditory meatus. They were modified in size and content in hyperplasia.

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Summario in Interlingua

Le Anatomia Microscopic del Aure Externe de Felis domesticus

Le aure de *Felis domesticus* esseva dividite in duo partes: Le pinna, con su centro de cartilagine elastic e le attachate musculos auricular, e le externe meato auditori.

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Usualmente le epidermis de ambe regiones esseva componite de solmente quatro stratos, i.e. le strato lucide esseva presente in casos rar solmente. Omne le stratos esseva usualmente tenue, sed in sectiones hyperplastic le strato cornee e le strato spinose cresceva marcatemente in spissitate durante que le strato granulose e le strato cylindric remaneva inalterate. Un basse numero de figuras mitotic esseva presente in le strato cylindric.

Le dermis del pinna esseva tenue e habeva dense tissu conjunctive, con pauc separation del fibras dermal. Illo cresceva in spissitate ab le puncta del pinna ad le externe canal auditori. Ambe lateres, le convexe e le concave, contineva glandulas sudorifere e sebacee con curte e amassate dispositivos excretori e secretori. Le distribution del capillo al latere convexe esseva usualmente dense. Le superficie concave habeva capillos sparse e fin, excepte verso le margine rostral. Le dermis del externe meato auditori habeva tanto un strato papillar del cute e etiam un strato reticular, sed le disposition del fibras esseva a vices alterate per le dimensiones e le numero del glandulas presente in le canal del aure. Le fibras del strato papillar del cute esseva micre e simile a illos in le pelle capillute. Le strato reticular deveniva dense ubi illo se fundeva con le perichondrio del cartilagine. Fibras elastic appareva in plus grande numeros e dimensiones in le tertio inferior del meato. Le numero del fibras esseva reducite in sectiones exhibiente hyperplasia.

Le glandulas sebacee mesurava inter 200 e 450 μ in diametro. A vices illos occurreva independentemente de lor folliculos capillar. Glandulas ceruminose esseva trovate in omne partes del externe meato auditori. Illos esseva modificate in lor dimensiones e in lor contento in hyperplasia.

Further Studies of Goat Serum Proteins by Paper Electrophoresis

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IN A RECENT STUDY of serum proteins of the goat by paper electrophoresis and chemical methods, Gorczyca *et al.*[2] encountered a wide distribution in the range of normal values. The limited appearance of the *beta*-2 globulin fraction in the female of the species was also commented upon.

Reasons for these variations in normal values and the limited appearance of the *beta*-2 globulin fraction were not established during the course of the study. Consideration was given the possibility that the age of goats used (7 to 9 months old), may have been a contributing factor in some undetermined manner. Consequently, it was decided that a study of serum proteins of older goats might supply additional information.

This report describes a study employing goats, 24 to 30 months of age, and provides a comparison of the groups studied.

MATERIALS AND METHODS

The 22 goats selected for this study consisted of purebreds and crossbreds of standard breeds, except the Angora. The group consisted of 14 females , 7 males, and 1 wether, 24 to 30 months old, with none of the females pregnant at the time of serum sampling. Allotment of the animals into three groups was based upon source and breed to facilitate data interpretation and comparison.

The first group, located in Dallas, Texas, and identified as the Texas goats, consisted of 1 wether, 5 female, and 2 male crossbred goats. The diet was the same as described in the original study.[2]

The second group, located in Chino, Calif., and identified as the California goats, consisted of 5 female and 4 male crossbred goats. The diet consisted of commercial meal* and alfalfa with no pasture.

Purebred Saanen goats comprised the third group consisting of 3 females and 2 males located in La Puente, Calif. The diet of this group was the same as that of the California goats.

Paper electrophoresis and chemical studies of serum samples were accomplished in the manner and by methods utilized in the original study by Gorczyca *et al.*[2] All determinations were accomplished in duplicate and found to reproduce within 1.5 per cent limits.

From the Clinical Laboratory of San Bernardino, San Bernardino, Calif. (Gorczyca and Lazaroni), and the Clin-Eval Farms, Inc., Alvarado, Tex. (McCarty).

* Purina chow, manufactured by the Ralston Purina Co., St. Louis, Mo.

RESULTS

The total protein concentration was determined chemically (table 1). Analytrol integration of paper electrophoresis fractionations is reported as the per cent of total protein for each respective component.

Results of comparative study of the 24- to 30-month-old goats are tabulated according to location, breed, and sex (table 2).

Comparisons of the respective age groups, sex groups, span of normal values, and the differences encountered between the respective means are presented (table 3).

The data (table 3) show statistically significant increases in total protein and *gamma* globulin concentrations in the older goats. A marked decrease is evident in the albumin entity.

The changes encountered between the age group means were reflected generally in sex group means. Males also had a significant increase in the *alpha* globulin concentration.

The *beta*-2 globulin shows an incidence of 25 per cent in the Texas goats, 60 per cent in the Saanen goats, and 100 per cent in the California goats.

Of considerable interest was the occurrence of a globulin fraction not previously encountered in our studies of goat serum proteins. This entity has a Rf value of 0.52, compared to albumin, which corresponds to the value previously reported for the *alpha*-2 globulin in human serum. Analytrol tracings of the electrophoretic protein fractionations of California goat 12 and Texas goat 5 are compared to show the three and five globulin fraction patterns (fig. 1).

DISCUSSION

In the original study of serum proteins of 7- to 9-month-old goats, a wide span was encountered in the normal concentrations of total protein and the respective protein components. This age group had been specifi-

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cally selected to preclude effects of variables such as mating, gestation, and lactation.

A similar wide span in the range of normals had been reported by Reineke *et al.*,[4] employing chemical salting-out procedures in studies of dairy goat proteins. No comparison or correlation of this data could be made with our results, since the age of the goats had not been reported in this early work.

Selection of 24- to 30-month-old goats for this study was based upon the conclusion that goats of this age could be considered to be in the prime of life and should, therefore, be more representative of the species than our original selection.

Generally, the means of the 24- to 30-month-old Texas goats and the respective sex groups indicated increased total protein and *alpha* and *gamma* globulin concentrations. No marked changes were noticed in the *beta*-2 globulin fraction. The albumin entity showed a marked decrease in concentration. Evaluation of the differences between the means show that significant changes were present in the *alpha* and *gamma* globulins in the three sets of means. Total protein showed a statistically significant decrease in the age group and female group means. The albumin change was actually of statistical significance in only the female group. The wethers were not included in the evaluation due to the limited number of samples available.

Comparison of the data pertaining to age groups seems to indicate that maturity produces some stabilization in the protein component concentrations. The ranges encountered in the older Texas goats generally indicated a more defined and delimiting distribution. Spans within the respective components ranged from 4 to 8 per cent. The results of this phase of the study seemed to indicate that the age of the animal was not a factor in presence and incidence of the *beta*-2 globulin.

At the conclusion of this phase of the study, transfer of the working site was made to California. To maintain a correlation with data pertaining to the older Texas goats, corresponding California crossbred goats were selected for comparative studies.

Comparison of the means of the two groups showed, in the California goats, a decrease in albumin and *beta*-1 globulin, increased *beta*-2 and *gamma* globulins, and the presence of a new entity, the *alpha*-2 globulin. These changes were generally reflected in the sex means, with statistical significance present in the changes encountered in the albumin, *beta*-2 and *gamma*

TABLE 1—Serum Protein Studies of Purebread and Crossbred Goats, 24 to 30 Months Old, Located in Texas and California

No.	Sex	Total protein (Gm./100 ml.)	Concentration (% of total protein)					
			Albumin	*Alpha*	*Alpha*-2	*Beta*-1	*Beta*-2	*Gamma*
TEXAS GOATS								
1	F	6.2	53.5	8.0		11.5	8.0	19.0
2	F	6.3	59.5	7.0		11.5	....	22.0
3	F	6.9	56.0	9.0		8.5	4.0	22.5
4	F	7.0	57.5	11.5		13.0	....	18.0
5	F	7.3	52.0	8.0		14.0	....	26.0
6	M	6.4	56.5	8.0		15.0	....	20.5
7	M	6.5	56.5	12.0		13.5	....	18.0
8	W	5.8	56.0	9.5		13.5	....	21.0
CALIFORNIA GOATS								
9	F	5.6	47.5	5.0		12.0	7.0	28.5
10	F	6.2	39.0	8.0		12.0	10.0	31.0
11	F	6.8	48.5	7.5	4.0	8.0	5.0	27.0
12	F	7.1	44.5	7.5	3.0	8.5	12.0	24.5
13	F	7.4	42.5	8.0		12.5	11.5	25.5
14	F	7.5	40.0	10.0		12.5	10.0	27.5
15	M	6.2	41.0	9.0		8.0	10.0	32.0
16	M	7.2	49.0	7.5		7.5	7.5	28.5
17	M	7.2	50.0	5.5	3.0	6.0	9.0	26.5
SAANEN GOATS								
18	F	6.6	54.5	7.5		10.0	10.5	17.5
19	F	7.1	46.0	9.5		11.0	6.5	27.0
20	F	7.4	51.0	8.0		18.0	....	23.0
21	M	6.8	44.5	9.5		19.5	....	26.5
22	M	7.1	40.0	8.5		15.0	11.5	25.0

TABLE 2—Comparison of Group Means, Sex Means, and Range of Normal Values in 24- to 30-Month-Old Goats

Group	No. of animals	Total protein (Gm./100 ml.)	Concentration (% of total protein)					
			Albumin	*Alpha*	*Alpha*-2	*Beta*-1	*Beta*-2	*Gamma*
GROUP MEANS								
*Texas	8	6.6	56.0	9.2		12.6	1.6	20.9
s.d.**		±6.6	±2.2	±1.7		±1.8		±2.5
Calif.	9	6.8	45.5	7.6	1.1	9.7	9.0	27.1
s.d.		±6.2	±4.0	±1.4		±2.4	±2.3	±2.5
Saanen	5	7.0	47.2	8.6		14.7	5.7	23.8
s.d.		±2.8	±4.9	±0.8		±3.0	±5.0	±3.4
FEMALE MEANS								
Texas	5	6.8	55.8	8.8	....	11.8	2.4	21.5
Calif.	6	6.8	43.7	7.6	1.1	10.9	9.2	27.2
Saanen	3	7.0	50.5	8.3	....	13.0	5.7	22.5
MALE MEANS								
Texas	2	6.4	56.5	10.0	....	14.3	....	19.2
Calif.	3	6.8	47.5	7.5	1.0	7.5	9.5	27.0
Saanen	2	7.0	42.3	9.0	....	17.2	5.8	25.7
RANGE OF NORMAL								
*Texas	8	5.8–7.3	52.0–59.5	7.0–12.0	.....	8.5–15.0	4.0– 8.0	18.0–26.0
Calif.	9	5.6–7.4	39.0–50.0	5.0–10.0	3.0–4.0	6.0–12.5	5.0–12.0	24.5–32.0
Saanen	5	6.6–7.4	40.0–54.5	7.5– 9.5	.....	10.0–19.5	6.5–11.5	17.5–27.0

* Wether data included. ** Standard deviation is shown as a per cent of total protein.

entities. The significant changes encountered generally augmented the initial changes seen in the older Texas goats in comparison to the 7- to 9-month-old Texas goats.

The 100 per cent incidence of the *beta*-2 globulin in California goats, as well as the appearance of the *alpha*-2 fraction, posed the question of environmental influence on results of this study. In an effort to resolve this problem, a third group of goats, consisting of 5 purebred Saanens, was selected for study.

Data pertaining to this group showed, in comparison to the California crossbreds, increased *beta*-1 and decreased *beta*-2 and *alpha*-2 globulins. These changes were due to decreased incidence of the *beta*-2 globu-

TABLE 3—Comparison of Means of the Age Groups, Sex Groups, and Range of Normal Values

Age (months)	No. of animals	Total protein (Gm./100 ml.)	Concentration (% of total protein)					
			Albumin	*Alpha*	*Alpha*-2	*Beta*-1	*Beta*-2	*Gamma*
GROUP MEANS								
7–9*	15	6.2	63.3	6.7	....	13.2	1.3	15.5
24–30	22	6.8	49.8	8.4	0.4	11.9	5.5	24.0
Difference**		9.6	−13.5	1.7	0.4	−1.3	4.2	8.5
FEMALE MEANS								
7–9	10	6.3	62.8	6.4	....	12.3	2.0	16.8
24–30	14	6.8	49.4	8.6	0.5	11.5	6.0	24.0
Difference		8.0	−13.4	1.2	0.5	−0.8	4.0	7.2
MALE MEANS								
7–9	3	5.9	62.5	5.8	....	17.0	....	15.1
24–30	7	6.8	48.2	8.6	0.4	12.0	5.4	25.4
Difference		15.1	−14.3	2.8	0.4	−5.0	5.4	10.3
WETHER MEANS								
7–9	3	6.2	60.0	9.4	....	16.6	....	14.2
24–30	1	5.8	56.0	9.5	....	13.5	....	21.0
Difference		−6.4	−4.0	0.1	....	−3.1	....	6.8
RANGE OF NORMAL								
Texas*		5.7–6.9	50.0–70.5	4.5–14.0	....	8.0–19.0	3.0– 8.5	5.0–23.0
Calif.		5.6–7.4	39.0–54.5	5.0–10.0	3.0–4.0	6.0–15.0	5.0–12.0	17.5–32.0
Texas		5.8–7.3	52.0–59.5	7.0–12.0	....	8.5–15.0	4.0– 8.0	18.0–26.0

* Values for 7- to 9-month-old goats were determined in an earlier study.[2] ** Differences are shown as percentages of total protein.

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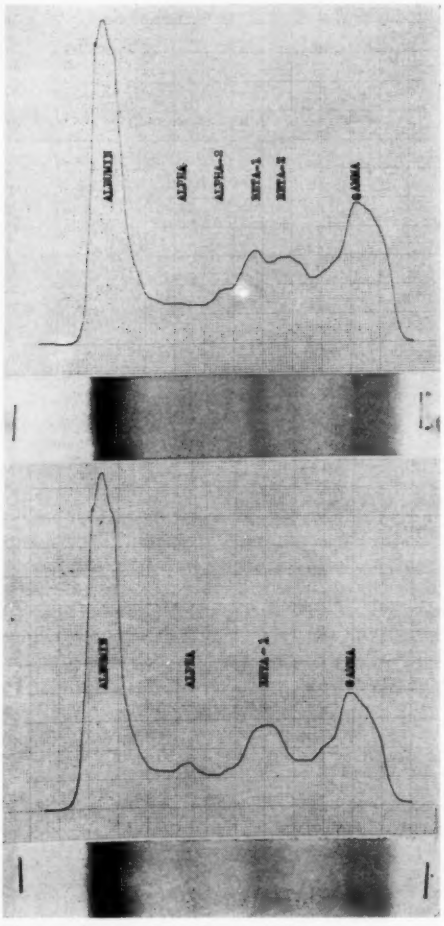

Fig. 1—Analytrol tracings of electrophoretic protein fractionations of California goat 12 (top) and Texas goat 5 showing the three and five globulin patterns.

lin and lack of appearance of the *alpha*-2 entity. Comparison of the means of the three groups of older goats places the Saanen group means between those of the Texas and California groups as pertains to presence and concentration of the respective protein components.

The results of this study indicate that changes of statistical significance are present in the individual group means, as well as in the composite group mean of the older animals in comparison to that of the younger goats. These changes are evident in increased total protein, *beta*-2 and *gamma* globulins, and markedly decreased albumin. Spans of the respective protein components, although more delimited in the older Texas goats, were found to range from 5 to 15 per cent in the California and Saanen groups. In recent work by Campbell,[1] similar wide spans in the respective protein components were encountered in paper electrophoretic studies of the cow, horse, sheep, pig, and dog, leading to the conclusion that this is a natural occurrence.

Establishment of reasons for the variable appearance of the *beta*-2 globulin will require further study. Gorczyca and McCarty,[3] in a study of prolonged low-dosage administration of antibiotics and superimposed *Candida albicans* infection on young goats, found that the *beta*-2 globulin appeared, if not present originally, and increased considerably in concentration as the infection proceeded to terminal stages, irrespective of the sex of the animal. It is evident from this information, as well as from the data in this study, that our original premise regarding confinement of this globulin entity to the female of the species was disproved.

Subsequent studies, in the process of completion, indicate that in some goats the *beta*-1 and *beta*-2 globulins are closely bound by undetermined factors and appear as a single entity. Serum specimens, stored at 10 C. for several weeks and subsequently re-run electrophoretically, showed a separation of this complex into the two globulin entities. Indications are that the *alpha*-2 globulin may also be a part of this complex; however, further work will be required to determine this fact due to the low incidence (5.7%) of this entity in goats studied.

Eletrophoretic studies have confirmed the presence of globulin fractions originally observed or postulated as present in goat serum by Reineke *et al.*,[4] However, it is evident that all the fractions do not necessarily appear in any individual goat.

It is hoped that further studies of purebred and crossbred goats will supply additional information of consequence on this subject. Such studies are now in progress.

SUMMARY

A study of serum proteins of 24- to 30-month-old goats was conducted by chemical and electrophoretic methods to elucidate reasons for the wide range in normal values

previously encountered in 7- to 9-month-old goats, and for the limited appearance of the *beta*-2 globulin in the female of the species. Results of this study were as follows:

1) Moderate to marked changes were found in mean total protein and protein component concentrations between the two age groups compared.

2) Goats of the older group had statistically significant increases in total protein and *beta*-2 and *gamma* globulins, with markedly decreased albumin concentration. This trend was also reflected in the means calculated according to the sex of the animals.

3) The range of the normal values was more defined in the older animals. In comparison with age means, a higher incidence of the *beta*-2 globulin was encountered in both sexes.

4) The occurrence of a globulin entity identified as *alpha*-2 globulin on basis of mobility was also found to concur with findings of earlier investigators.

5) Significant variations, based on location and breed, were found in total protein and protein components of the groups of older goats.

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SUMMARIO IN INTERLINGUA

Studios Additional de Proteinas Seral del Capra, Effectuate per Medio de Electrophorese a Papiro

Le proteinas seral in capras de etates de inter 24 e 30 menses esseva studiate per medio de methodos chimic e electrophoretic, con le objectivo de elucidar le rationes pro le extense gamma de valores normal previemente incontrate in capras de etates de inter 7 e 9 menses e pro le basse incidentia de globulina beta-2 in le femininas del specie. Le resultatos del studio esseva le sequentes:

1. Moderate o marcate alterationes esseva trovate in le concentrationes medie de proteina total e de componentes individual de proteina in comparationes del duo gruppos de etate.

2. Capras del gruppo de etate plus avantiate monstrava statisticamente significative augmentos de proteina total e de globulina beta-2 e gamma, con marcate reductiones del concentration de albumina. Iste trend esseva etiam reflectite in le valores medie calculate separatemente pro le duo sexos.

3. Le gamma de normalitate esseva melio definite in le valores obtenite ab le animales plus matur. In comparation con le valores medie pro le gruppos de etate individual, le incidentia de globulina beta-2 se monstrava plus alte in le gruppos plus matur in ambe sexos.

4. Le occurrentia de un entitate globulinic identificate como globulina alpha-2 super le base de su motilitate esseva etiam trovate documentate per le constatationes de previe investigatores.

5. Significative variationes esseva constatate inter differente regiones geographic e inter differente racias in le valores pro proteina total e pro le componentes de proteina in le caso del gruppos de capras de etate plus matur.

Neurotropism of Equine Influenza-Abortion Virus in Infant Experimental Animals

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THE PROPERTIES of equine influenza virus (EIV) and of equine abortion virus (EAV) have been studied comprehensively in horses,[4] laboratory animals,[1,2] chicken embryos,[3] and tissue cultures.[6,7] An adaptation of EIV (Army 183 strain) to infant mice by the intracerebral route has recently been reported.[5]

A series of experiments, conducted in this laboratory since 1953 on infant kittens, mice, and hamsters, revealed that EIV and EAV possess neurotropic properties.

Analysis and discussion of the findings obtained from a study of the histopathologic lesions in the central nervous system (CNS), produced by the use of several strains of EIV and EAV by various routes of inoculation, is the purpose of this report.

MATERIALS AND METHODS

Thirty-eight 1-day-old cats, nine 1-day-old and eleven 20-day-old white mice, and fifty-eight hamsters were used in this experiment (table 1).

The cats were simultaneously inoculated intraperitoneally and intranasally with the Grayson I strain of EIV* at the rate of 1.0 ml. and 0.5 ml. of the filtrate, respectively. A 30 per cent suspension of virulent lung and spleen tissue of this strain in sodium chloride solution was used as the initial inoculum. In the successive passages, spleen and lung tissues from the infected cats were used in a similar manner to prepare inoculum; the same doses and routes were used.

Eleven of the white mice used belonged to a group of infant mice to which the Army 183 strain of EIV had been adapted by the intracerebral route.[5] Their passage levels ranged from the 36th to the 57th. The other mice used were 1-day-old white mice and were also inoculated intracerebrally with the LSSSL 491 strain* of EAV.

Of the hamsters used, 12 were inoculated intraperitoneally with the Army 183 strain and represented animals of the early passages (1–6) for visceral adaptation. The 46 remaining were inoculated with LSSSL 491 strain (6 intraperitoneally, 14 intranasally, 17 intracerebrally, and 9 intramuscularly).

Most of the animals were moribund when killed and specimens from all were immediately fixed in Zenker's fluid. Sections (5 μ) were routinely stained with Harris' hematoxylin and aqueous eosin. In addition, Shorr and Giemsa techniques were employed when necessary.

A virus-neutralization test for LSSSL 491 was conducted with serum from a horse which had recovered from equine influenza.

RESULTS

After an incubation period of four to five days, the cats showed certain clinical manifestations of virus encephalomyelitis (clonic spasms, lack of orientation, repeated falls) which, in almost all instances, terminated in paralysis, coma, and death within 24 to 48 hours from the onset of signs. Necropsy usually revealed meningeal congestion, petechial hemorrhages, and edema of the brain. Often the lateral ventricles were enlarged. Petechial hemorrhages of the thymus and of the thoracic and abdominal organs, pneumonia, necrotic hepatitis, splenitis, and pleural and peritoneal effusions were additional findings. The intensity and distribution varied in each kitten.

Microscopically, the lesions of the visceral organs (liver and lung) were similar to those described by others in relation to equine fetuses aborted because of EAV infection (fig. 1, 2). In addition, there were characteristic lesions in the nasal cavities which contained mucopurulent material. Numerous necrotic foci were dispersed in the desquamated or eroded mucosa. The lamina propria and submucosa were intensely inflamed and hyperplastic. In the proliferated epithelium of the regio olfac-

From the Live Stock Sanitary Service Laboratory, University of Maryland, College Park.

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* This strain was obtained by Col. H. J. Kintner from a sick horse at a Florida race track.

* This strain was isolated from an aborted equine fetus brought to the Live Stock Sanitary Service Laboratory in 1958.

TABLE 1—Data on Results Obtained in Experimental Animals Inoculated with Equine Influenza Virus (EIV) and Equine Abortion Virus (EAV)

Species	No. of animals used	Virus	Strain	Inoculation route	Passage level	Lesions	
						Visceral	C.N.S.
Kittens (1 day old)	38	EIV	Grayson I	Intraperitoneal and intranasal simultaneously	1st–7th	+++	+++
Mice (infant)	11	EIV	Army 183	Intracerebral	36th–57th	–	+++
Mice (1 day old)	9	EAV	LSSSL 491	Intracerebral	1st	–	+++
Hamsters (20 days old)	12	EIV	Army 183	Intraperitoneal	1st–6th	+++	++
Hamsters (1 day old)	6	EAV	LSSSL 491	Intraperitoneal	1st	++	+++
	14	EAV	LSSSL 491	Intranasal	3rd	–	+++
	17	EAV	LSSSL 491	Intracerebral	2nd	–	+++
	9	EAV	LSSSL 491	Intramuscular	2nd	+	+++

toria and at the margins of the necrotic areas, the cells had typical eosinophilic intranuclear inclusion bodies (fig. 3). In the brain and spinal cord, the lesions consisted of meningeal infiltration, capillary congestion, endothelial cell proliferation, tiny hemorrhages, perivascular cuffing, scattered focal necrosis, inflammatory nodules, and neuronal degeneration. Occasionally there was destruction, demyelinization of nerve pathways, gliosis and, finally, ependymal cell proliferation or degeneration, or both. The perivascular cuffings were usually composed of lymphocytes, presumably glial cells, and a few granulocytes. The nodules were rounded collections of glial and hematogenous cells in the center of which cellular detritus or a small capillary could occasionally be identified. The neuronal degeneration consisted of ballooning, chromatolysis, or pyknosis with or without satellitosis. These lesions varied in extent and intensity; they were noticeable in the olfactory lobe and severe in the thal-

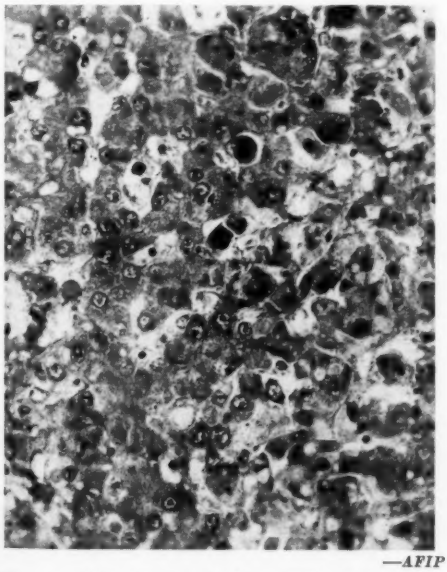

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Fig. 1—Liver of a cat given EIV of the third passage level. Numerous intranuclear inclusion bodies in the parenchymatous cells at the edge of a necrotic area are shown (lower). H & E stain; x 338.

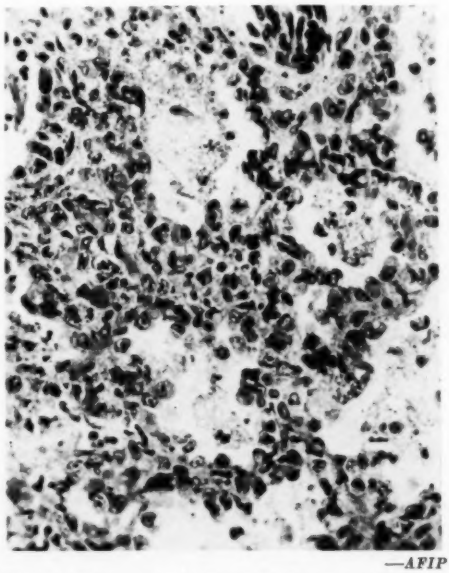

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Fig. 2—Lung of cat shown in figure 1. Alveolar cells with characteristic intranuclear inclusion bodies are seen in the vicinity of necrotic areas. H & E stain; x 338.

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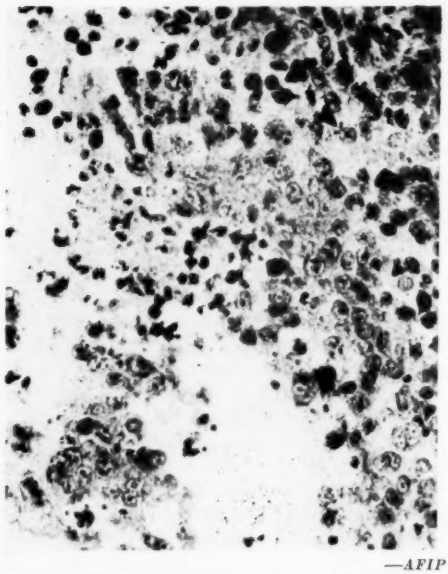

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Fig. 3—Cat given EIV, fifth passage level. Nasal turbinates have hyperplasia of the epithelium with focal necrosis, neutrophilic infiltration, and cellular detritus. Numerous cells of the olfactory epithelium show characteristic intranuclear inclusion bodies. H & E stain; x 385.

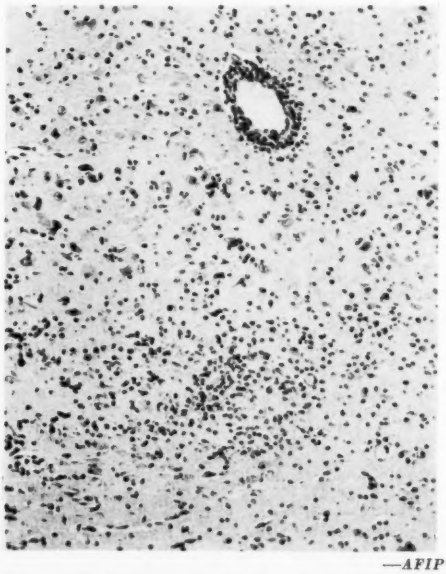

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Fig. 4—Thalamic area of a cat given EIV of the third passage level. Prominent perivascular lymphocytic infiltration, neuron degeneration and destruction, diffuse gliosis. H & E stain; x 120.

amus, medulla oblongata, and in the periventricular areas (fig. 4–7). Lesions were more severe in the animals at the second to the fifth passage levels. In the nerve cells were characteristic intranuclear inclusion bodies which ranged in size from that of a small pinhead to ones filling the entire nucleus. They were usually eosinophilic and similar to those in the epithelial cells of the liver and lungs. Occasionally, these bodies were in the small neurons, but more often in the astrocytes and microglial cells. They were also seen in the ependymal cells, adjacent to the necrotic areas, but were absent

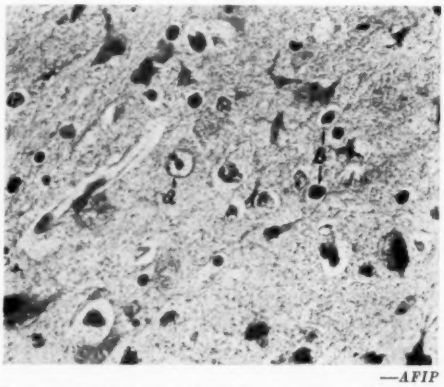

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Fig. 5—Higher magnification of area in figure 3. Notice intranuclear inclusion bodies in the neuron (a) and microglia (b). H & E stain; x 326.

from the large motor cells of the medulla oblongata and spinal cord.

The 11 white infant mice to which the Army 183 strain was adapted showed clinical manifestations and lesions of acute nonsuppurative encephalomyelitis with inclusion bodies similar to those mentioned previously (fig. 8). Also, there were well-defined areas of liquefaction necrosis, leaving a reduced amount of stroma with scattered isolated nerve cells, some of which had characteristic inclusion bodies. Unlike the lesions found in the cats, none of the lesions in the mice were in the visceral organs or nasal cavities.

The 9 white mice inoculated with the LSSSL 491 strain of EAV (previously passed through the brains of hamsters at a third passage level) also developed extensive virus encephalomyelitis, but only a few inclusion bodies were found.

The hamsters to which the Army 183 strain was adapted by intraperitoneal in-

jections (visceral adaptation) had characteristic lesions and intranuclear inclusion bodies in the cells of the visceral organs. In general, this was also true for the cats and aborted fetuses of affected mares, but the lesions in the hamsters were those of encephalomyelitis in an early stage, including mild cellular infiltration in the Virchow-Robin spaces and, in a few instances, small intranuclear inclusion bodies in the neuroglial cells. Presumably, with these

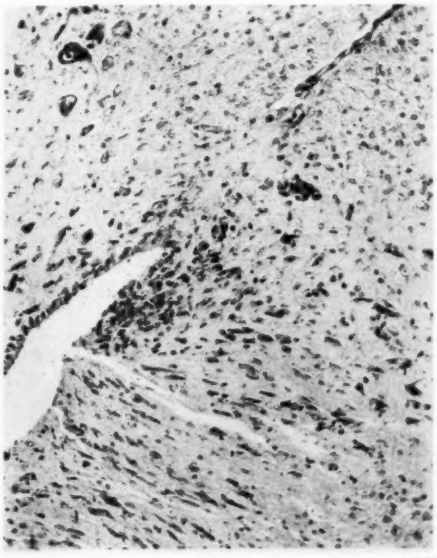

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Fig. 6—Spinal cord of a cat given EIV of the fourth passage level. Notice small vessel with perivascular lymphocytic infiltration and glial nodule in the periependymal area. Giemsa stain; x 150.

successive passages for visceral adaptation in which death occurred within 48 hours, the lesions of the CNS lacked time to develop completely.

The rest of the hamsters, inoculated by various routes with the LSSSL 491 strain of EAV, developed clinical manifestations of CNS disease and lesions of severe encephalomyelitis with pathognomonic inclusion bodies in the nerve cells (fig. 9–11). It is noteworthy that, in this group, the first 6 hamsters inoculated intraperitoneally had only moderate lesions in the visceral organs and no inclusion bodies, but conspicuous lesions were found in the CNS. It was from the brains of these 6 hamsters that inocu-

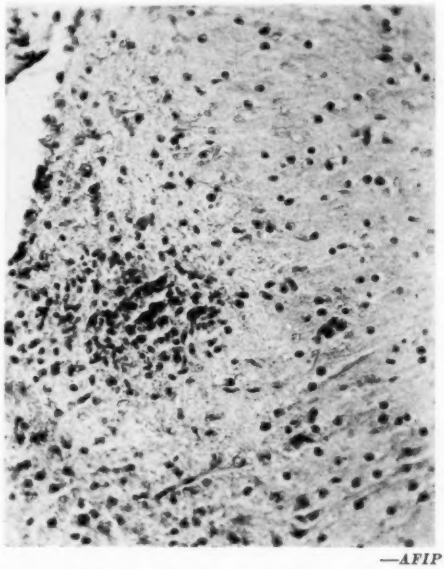

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Fig. 7—Spinal cord of a cat given EIV of the sixth passage level. Glial nodule in the fasciculus cuneatus with intranuclear inclusion bodies in the microglia. Giemsa stain; x 230.

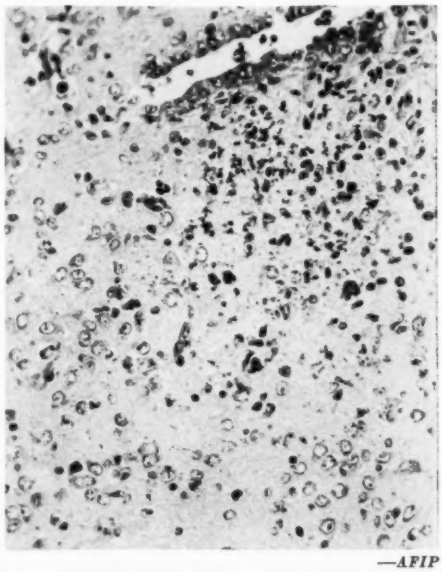

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Fig. 8—Mouse given fourth passage. Large necrotic area adjacent to the lateral ventricle with neutrophilic infiltration and cellular debris. Many of the neuroglia show intranuclear inclusion bodies. H & E stain; x 230.

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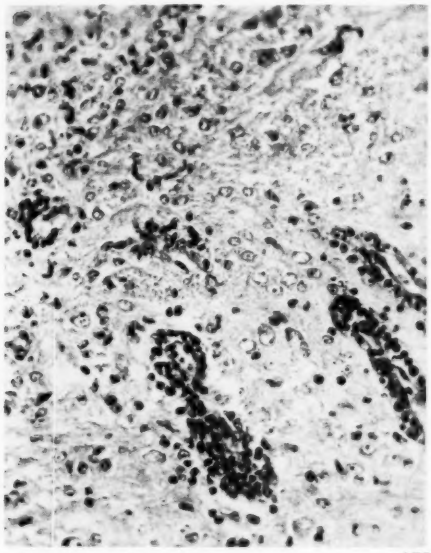

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Fig. 9—Hamster inoculated intraperitoneally with EAV strain 491 of the first passage has heavy perivascular infiltration, necrotic area in the medulla, and numerous neuroglia cells with characteristic intranuclear inclusion bodies. H & E stain; x 230.

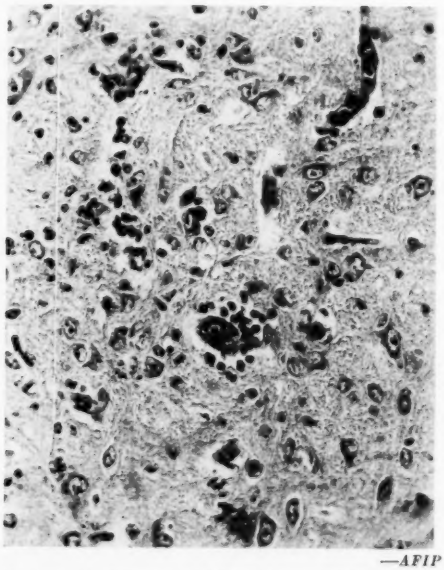

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Fig. 10—Hamster inoculated intracerebrally with EAV strain 491 of the second passage level has thrombosis of the capillaries and perivascular infiltration; many of the neuroglia have characteristic intranuclear inclusion bodies. H & E stain; x 230.

lum was prepared and used for the rest of this group.

The serologic test of the hamsters given the final passage showed that the immune equine serum completely neutralized the LSSSL 491 strain, while normal horse serum did not.

DISCUSSION

It is evident that 1-day-old kittens are susceptible to experimental infection with EIV.

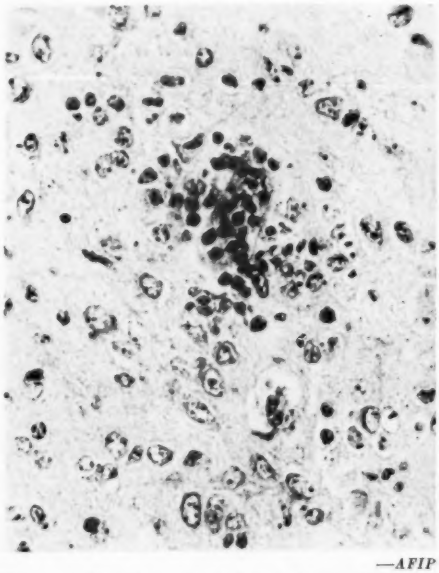

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Fig. 11—Hamster inoculated intranasally with EAV strain 491 of the second passage level has a characteristic lesion of virus encephalomyelitis. There are numerous neurons with intranuclear inclusion bodies. H & E stain; x 440.

The involvement of the CNS and the lesions of the olfactory lobe in the cats inoculated simultaneously by the intranasal and intraperitoneal routes indicate the predilection and the path of the EIV; *i.e.*, through the rhinencephalon, along the olfactory tract, to the main portion of the brain. It is also evident that, in the hamsters inoculated intranasally only, the EAV reaches the brain through the same path. This route of propagation is probably similar to that encountered in experimental and spontaneous *Listeria monocytogenes* infection and in neurotropic virus diseases.

In addition, 1-day-old mice are suscep-

tible to intracerebral EAV infection and the histopathologic lesions of the CNS are identical to those found in infant mice to which the Army 183 strain of EIV has been adapted.

Another characteristic of EAV is its property of attacking the CNS of suckling hamsters and producing identical lesions, not only when inoculated intracerebrally or intranasally, but also intraperitoneally and intramuscularly. It is obvious, therefore, that the pattern of the development of the clinical manifestations and the histopathologic changes produced by EIV and EAV in these animals are identical. In the cats and hamsters, it appears that the presence of the lesions in the visceral organs depends to a certain extent on the route used for the penetration of the virus, whereas lesions in the CNS always appear, regardless of the route used. This point, however, is only partially true with regard to young hamsters to which the Army 183 strain was used for visceral adaptation. Presumably, with these successive passages for visceral adaptation in which death occurred within 48 hours, lesions in the CNS lacked time to develop completely. In addition, the factor of the animal's age is also to be considered. It is probable that the rapid expansion and development of the CNS of mammals shortly after birth may constitute a favorable condition for virus development.

The similarity of lesions in the brain of the three animal species (cats, mice, and hamsters), produced by different strains and routes, provides evidence of the neurotropism of EIV and EAV in the infants of these species and also confirms the concept of the single identity of these viruses.

Knowledge of the neurotropism of equine influenza-abortion virus in infant experimental animals may be valuable when exploring the possibility of virus propagation in the brains of other experimental infant animals.

SUMMARY

Infant cats, white mice, and hamsters inoculated by various routes with equine influenza-abortion virus consistently developed lesions of encephalomyelitis with characteristic intranuclear inclusion bodies in the neuroglial cells. Characteristic lesions in the visceral organs or the nasal epithelium, or both, coexisted with the cerebral lesions only when the virus was introduced by the peritoneal or nasal routes, or both.

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SUMMARIO IN INTERLINGUA

Neurotropismo de Virus de Influenza Equin e de Virus de Abortion Equin in Neonate Animales Experimental

Neonate cattos, muses blanc, e hamsters, quando inoculate per varie vias con virus de influenza e abortion equin, disveloppava uniformemente lesiones de encephalomyelitis con characteristic corpores de inclusion intranuclear in le cellulas neuroglial. Characteristic lesiones in le organos visceral o in le epithelio nasal o in ambes co-existeva con le lesiones cerebral solmente quando le virus esseva introducite per via peritoneal e/o nasal.

XUM

Thyroidal Iodine^{131} Turnover, Thyroxine Secretion Rate, and Thyroactive Iodinated Casein Utilization in Mink (*Mustela vison*)

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ALTHOUGH MINK (*Mustela vison*) are raised extensively because of their value as fur bearers, little information is available on their endocrine balance. Such information would be of interest in comparative endocrinology and might also be useful in elucidating the role of the endocrine system in such processes as reproduction and fur growth in this species. The present study was undertaken to measure some of the parameters of thyroid function in mink.

MATERIALS AND METHODS

Fifteen male mink, approximately 10 months old and weighing 1,000 to 1,500 Gm. (they had been reared on experimental diets), were allotted to three groups of similar average weight. The experiments were designed so that all animals could be used initially for measurement of the 24-hour uptake of iodine^{131} by the thyroid. Those in group I were then used to determine the thyroidal I^{131} output rate; those in group II, the thyroxine secretion rate; and those in group III, the utilization of thyroxine from orally administered iodinated casein. One week before beginning the experiments, all animals were started on the ration shown (table 1). They were maintained outdoors in individual wire cages containing nest boxes for protection from the weather. During the last two weeks of April, when the measurements were taken, mean daily temperatures ranged from 36 to 48 F., and averaged 43 F.

TABLE 1—Composition of Mink Ration

Ingredient	%
Horsemeat, with bone	21⅔
Fish (ocean whiting)	21⅔
Tripe	21⅔
Fresh liver	5
Cooked cereal mix (vitamin-supplemented)	30

L-thyroxine, used as a standard for thyroid secretion rate determination, was purified as described earlier.[7] A carefully weighed quantity was dissolved in dilute NaOH and then the solution was adjusted with dilute HCl to the point where a barely discernible precipitate began to form. After being made up to the required volume, this stock solution was used to prepare a series of solutions of progressively increasing concentration, so that at all thyroxine levels 0.01 ml. was given per 10 Gm. of body weieght. Injections were given subcutaneously once daily. Thyroactive iodinated casein,* standardized to contain 1.0 per cent true thyroxine, was suspended by grinding in a mortar with distilled water and a minimum quantity of Na_2CO_3 solution. The desired quantity was given daily by mixing with a small portion of mink food. No more food was given until this had been consumed.

In vivo thyroid and body counts were taken under pentobarbital sodium anesthesia (20 to 30 mg./kg.) by use of a scintillation counter and count rate meter. The scintillation detector was shielded by mounting it beneath an aperture (1-inch diameter) located 4 inches from one end of a 1 by 10 by 14-inch lead plate. Counts for the thyroid and body counts over the epigastric region were taken by centering the respective areas over this aperture.

Each mink was given 30 μc. of carrier-free I^{131} subcutaneously. Thyroid counts were taken daily for the first two days and at two-day intervals thereafter. Thyroid and I^{131} standards were corrected by the appropriate background, and thyroid uptake, expressed as per cent of injected dose, was computed for all mink. Thyroid output rates (group I) were computed by the usual method.[10] Thyroid secretion rate (group II) and the equivalent value for iodinated casein (group III) was estimated by the procedure first reported for the rat by Reineke and Singh.[7] The thyroxine or thyroactive iodinated casein dose for each animal was increased progressively at two-day intervals, a radioactivity count being taken before each increase in hormone dose. The per cent of the I^{131} dose retained in the thyroid at each count was expressed as the per cent of the preceding value and equated against dosage. Thyroid secretion rate was taken as the point where "per cent preceding count" reached 100.

RESULTS

I^{131} Uptake and Output Rate.—Maximum I^{131} uptake by the thyroid gland occurred

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* Thyroprotein, supplied by Agri-Tech. Inc., Kansas City, Mo.

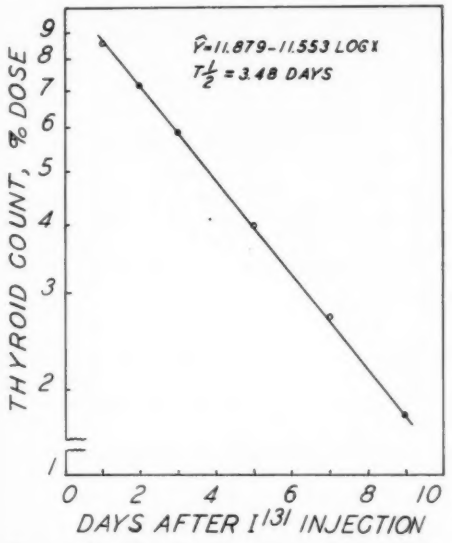

Fig. 1—Thyroidal I^{131} output curve of normal male mink. Each point is the average for 5 animals.

in all mink within 24 hours. The 15 mink had a mean 24-hour uptake of 8.14 ± 0.315* per cent of the dose. Individual values ranged from 7.73 to 10.07 per cent.

Daily values for 5 control mink (group I), taken over a period of nine days, were averaged and the regression between per cent of the dose and time was computed (fig. 1). The mean output rate was 19.9 per cent per day, and mean output 1/2 time (T 1/2) was 3.48 days. The T 1/2 values for individual mink ranged from 2.48 to 4.34 days.

Thyroxine Secretion Rate and Iodinated Casein Equivalent.—The data for groups II and III mink were plotted on separate charts and the response lines were fitted by the method of least squares. The broken lines represent the standard error of y, where y is the per cent preceding count. An estimate of precision of such assays [2] in terms of x, the thyroxine dosage, is obtained by the expression $\lambda = \frac{8y}{b}$, where Sy = standard error of the estimate, and b = slope. In the results given, λ is used as the error term.

The average thyroid secretion rate of 5 male mink (fig. 2) was 0.946 ± 0.098 γ L-thyroxine daily per 100 Gm. of body weight. The dosage of thyroactive iodinated casein required to reach the same end point was 0.276 ± 0.034 mg. daily per 100 Gm. of body weight (fig. 3).

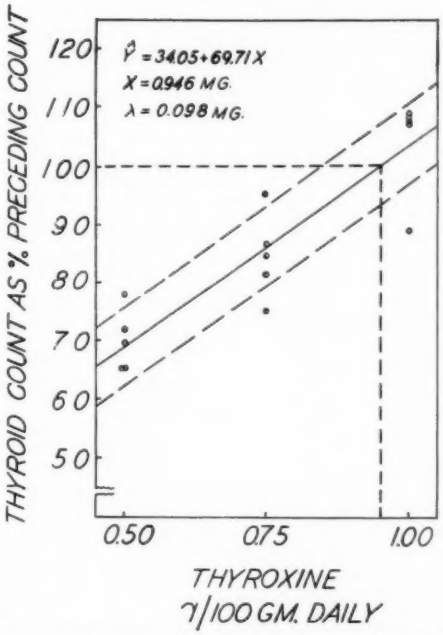

Fig. 2—Response of mink thyroid to injected thyroxine. Thyroid secretion rate is taken as the point where thyroid count reaches 100 per cent of the preceding count.

If it is assumed that the true thyroxine secretion rate of the mink in the two groups is actually the same, these values can be used to estimate the amount of orally available thyroxine in thyroactive iodinated casein. The casein contained 1.0 per cent thyroxine. A daily oral dose of 0.276 mg., containing 2.76 γ thyroxine, elicited a response equivalent to that of 0.946 γ of injected thyroxine. Therefore, the utilization of thyroxine in thyroactive iodinated casein was:

$$\frac{0.946}{2.76} \times 100 = 34.3\%$$

DISCUSSION

The thyroidal I^{131} output rate of mink (T 1/2 = 3.48 days) is nearly identical to

* Standard error.

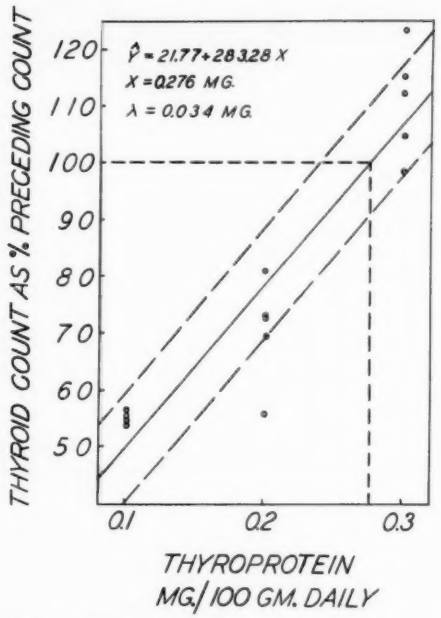

Fig. 3—Response of mink thyroid to oral thyroactive iodinated casein.

that of rats [9] and mice,[3] and much faster than in goats,[4] sheep,[5] or cattle.[8] Thyroidal I^{131} uptake is also within the range observed in rats and mice under ordinary laboratory conditions.

The thyroid secretion rate of 0.95 γ L-thyroxine/100 Gm. daily in mink compares with values of 2.69 γ and 2.15 γ in the mouse [1] and rat,[7] respectively.

As pointed out elsewhere, thyroid secretion rate would be expected to vary with metabolic body size rather than simple body weight.[6] A tabulation of thyroid secretion rate values for several species of animals varying widely in body size shows a range from 2.69 γ/100 Gm. for mice to 0.32 γ/100 Gm. for sheep. The daily amount of thyroxine secreted was divided by body weight raised to the 0.73 power to adjust the values for metabolic body size. By this calculation, values for all species from mice to goats had thyroid secretion rates of the same order of magnitude, ranging from 9.1 to 16.1 γ per kilogram$^{0.73}$. The mink in the present study, averaging 1,190 Gm., had a thyroid secretion rate of 0.95 γ thyroxine/100 Gm. or 11.26 γ daily per animal. This is equivalent to 9.92 γ kilogram$^{0.73}$, a value well within the range for the other species thus far studied by the same method.

The comparison between injected thyroxine and oral thyroactive iodinated casein indicates that about ⅓ of the thyroxine in the casein is available to the mink by this route. Whether the apparent loss in activity is due to incomplete digestion or absorption, or partial inactivation of thyroxine in the digestive tract, cannot be determined from the information available at present.

SUMMARY

The mean 24-hour thyroidal uptake of I^{131} by 15 10-month-old male mink was 8.14 ± 0.315 per cent of the injected dose. Peak uptake of I^{131} by the thyroid was reached in 24 hours. Thyroid output, determined in 5 mink, was at the rate of 19.9 per cent per day (T 1/2 = 3.48 days). The mean thyroxine secretion rate of 5 male mink, determined by thyroxine substitution, was 0.95 γ/100 Gm. per day or 9.92 γ daily per kilogram$^{0.73}$. When thyroprotein that contained 1.0 per cent thyroxine was given orally, 0.276 mg./100 Gm. per day provided available thyroxine equivalent to the normal secretion rate. From this comparison, it was computed that 34.3 per cent of the thyroxine in thyroactive iodinated casein protein is absorbed by mink when given orally.

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SUMMARIO IN INTERLINGUA

Le Metabolismo Thyroidic de Radio-Iodo (I^{131}), le Intensitate del Secretion de Thyroxina, e le Utilisation de Iodate Caseina Thyroactive in Visones (Mustela vison)

In 15 visones mascule de 10 menses de etate, le captation thyroidic medie de I^{131} in 24 horas esseva 8,14 ± 0,315 pro cento del dose injicite. Le intensitate maximal del captation de I^{131} per le thyroide esseva attingite in 24 horas. Le liberation thyroidic, determinate in 5 visones, progredeva con un intensitate de 19,9 pro cento per die, i.e. le tempore del medie valor esseva 3,48 dies. Le secretion medie de thyroxina, determinate in 5 visones mascule per medio de substitution de thyroxina, amontava a 0,95 gamma per 100 g per die o 9,92 gamma per die per kg elevate al potentia 0,73. Quando thyroproteina, continente 1,0 pro cento de thyroxina, esseva administrate per via oral, 0,276 mg per 100 g per die resultava in un disponibilitate de thyroxina equivalente al secretion normal. Ab iste comparation, il esseva calculate que 34,3 pro cento del thyroxina in iodate proteina thyroactive de caseina es absorbite per le visones quando illo es administrate oralmente.

Blood Enzyme Interrelationships in White Muscle Disease

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BLOOD ENZYME LEVELS may provide information relative to the nature of a disease as well as serving as diagnostic aids. For example, increased levels of serum glutamic oxalacetic-transaminase (SGOT) or lactic dehydrogenase indicate cellular destruction and are used as diagnostic aids in human myocardial infarctions.[11, 7] Little information is available concerning blood enzyme levels in animal diseases.

The SGOT level was found to increase greatly in white muscle disease (nutritional muscular dystrophy) of ruminant animals.[5] The increased concentration of this enzyme has been used in the diagnosis of white muscle disease by Kuttler and Marble [10] and by Swingle *et al.*[14]

The purpose of this study was to study the interrelationships among several blood enzymes in white muscle disease. The enzymes studied were lactic dehydrogenase, SGOT, alkaline phosphatase, acid phosphatase, and true cholinesterase. These five enzymes are those most frequently used as diagnostic aids in human diseases.

METHODS AND MATERIALS

Control blood samples were obtained from Rambouillet, Columbia, and Hampshire lambs in a University of Nevada flock. Two categories of lambs with white muscle disease were studied: 1) those with spontaneous white muscle disease, occurring on several ranches in western Nevada; and 2) those with muscular dystrophy induced by feeding a cod-liver oil diet.[10] The spontaneous cases were in spring lambs from ewes wintered on locally grown hay, usually without supplementation. Lambing occurred before the ewes were placed on pasture. The induced cases were in young lambs weaned from the ewes and put on a skim milk-cod liver oil ration.

Naturally affected lambs were studied first and, if enzyme levels were significantly different from the control level, further studies were made with blood samples from lambs with induced muscular dystrophy. Several studies were also made of the influence of normal variables, such as breed, on the blood levels of various enzymes. For these studies, normal control lambs were used.

The SGOT level was determined colorimetrically.[1] For this test, the oxalacetate formed upon incubation of the serum with *alpha*-ketoglutarate and L-aspartate was measured by forming the colored hydrazone. The units used are equivalent to those of the more precise Karman ultraviolet method.[9]

Serum lactic dehydrogenase was determined by the colorimetric method of Berger and Broida.[3] In this procedure, the serum is incubated with pyruvic acid in the presence of reduced diphosphopyridine nucleotide (DPNH) and the amount of pyruvic acid remaining is measured after formation of the colored hydrazone.

Acid and alkaline phosphatase were measured at pH 4.8 and 10.3, respectively, using p-nitrophenyl phosphate as the substrate. The method was that of Bessey *et al.*[4]

A few determinations of true cholinesterase were made using the method of Caraway.[6] This method photometrically measures the rate of pH change, using an acetylcholine substrate.

Conventional, statistical techniques were used in the analysis of the data.[13] Tests of significance were made using the Student's "t" test. Results of factorially designed studies were subjected to the conventional analysis of variance procedures. Where regression equations were computed, the data were fitted by the method of least squares. Reference will be made to various other statistical methods in discussion of the results of this study.

RESULTS AND DISCUSSION

The standard deviation of analysis for the various enzymes was determined from duplicate sample analysis.[15] These analytical errors (table 1) give an indication of the precision of the methods used.

To determine if it was necessary to pair experimental and control animals, a factorial study was made of the effect of breed, sex, and age on the blood SGOT and lactic dehydrogenase. Three breeds (Rambouillet, Columbia, and Hampshire) and both sexes were studied at 7 and 35 days of age. These ages were chosen because they included the ages of most of the lambs with spontaneous white muscle disease. The transaminase experiment had four replications per category and the lactic dehydrogenase experiment had two. None of the

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factors studied significantly influenced the blood serum lactic dehydrogenase or transaminase; hence, it was acceptable to use animals selected at random for the remainder of the experimental work.

TABLE 1—Precision of Analytical Methods Used

Constituent	Standard deviation	
	units/ml.	% of mean
Glutamic-oxalacetic transaminase	4.4	6.7
Lactic dehydrogenase	150.0	10.5
Alkaline phosphatase	0.14	1.0

It was felt that some advantage might be gained by using twin lambs in the studies of induced white muscle disease. The SGOT levels of a group of twin lambs were studied by the method of partial correlation.[13] It was found that, with respect to transaminase, twin lambs were no more similar than lambs selected at random.

The data from lambs with spontaneous white muscle disease and control data are presented (table 2). The lactic dehydrogenase increased markedly in lambs with white muscle disease. The alkaline phosphatase decreased to about half its control value, and the acid phosphatase was unaffected by white muscle disease. Comparison of the standard deviations of the mean values (table 2) with the analytical errors (table 1) indicates that the standard deviation of the mean arises almost entirely from biological variability rather than analytical error.

The increased lactic dehydrogenase levels found in lambs with white muscle disease was of the same order as that for SGOT. It will be noticed that serum lactic dehydrogenase and transaminase presumably have a common origin in the destruction of tissue cells. To ascertain if a relationship existed between the activities of these two enzymes in blood, a group of blood samples from lambs with induced muscular dystrophy were studied. These samples were selected so as to have the greatest possible range of transaminase levels. The data are presented (fig. 1). A linear correlation was found between the SGOT and lactic dehydrogenase. The correlation was very highly significant, with a correlation coefficient of +0.97 over 28 degrees of freedom. Although the correlation is linear, figure 1 is a logarithmic plot, since this type of graph is well adapted to representing wide ranges of data and errors are represented by the same linear distance everywhere on the plot.

The correlation of SGOT and lactic dehydrogenase indicates that they are released from damaged muscle tissue in equivalent amounts. Hence, the release of these enzymes is a part of a general breakdown of the affected cells rather than a specific excretion of either. In view of the correlation between these two enzymes, either could be used as a diagnostic aid in white muscle disease. The two analyses are equally difficult, but the reagent cost for the determination of lactic dehydrogenase is much greater than for transaminase.

TABLE 2—Blood Serum Enzymes in White Muscle Disease

Constituent	Units per milliliter						Significance of difference, P
	White muscle disease			Control			
	No.*	Mean	s.d.†	No.*	Mean	s.d.†	
Glutamic-oxalacetic transaminase [a]	17	1,290.0	1,310.0	31	56	31	0.001
Lactic dehydrogenase	10	22,310.0	10,000.0	21	1,152	297	0.001
Acid phosphatase	11	0.38	0.16	13	0.33	0.08	.....
Alkaline phosphatase	15	5.4	1.9	13	10.5	3.7	0.001

* Number of lambs. † s.d. = standard deviation.

In spontaneous white muscle disease, the alkaline phosphatase decreased to about half its normal value (table 2). In an attempt to establish a relationship between alkaline phosphatase and SGOT, analyses were conducted on a set of blood samples from lambs with muscular dystrophy induced by feeding cod liver oil.[10] As with the lactic dehydrogenase study, the samples were selected to give as wide a spread of transaminase levels as possible. No simple relation could be found between these enzymes. Alkaline phosphatase did not decrease until the SGOT level became quite high (table 3).

Proven decreases in alkaline phosphatase with disease are rare. Alkaline phosphatase increases in many conditions associated

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TABLE 3—Blood Serium Alkaline Phosphatase in White Muscle Disease

Condition	No. of lambs	Transaminase range (units/ml.)	Alkaline phosphatase (units/ml.)
Control	13	.....	10.5 ± 3.7
Spontaneous white muscle disease	15	.....	5.4 ± 1.9*
Induced muscular dystrophy	10	200–1,000	10.8 ± 6.2
Induced muscular dystrophy	11	Over 1,000	6.3 ± 1.5*

* These values are significantly different from control values (P less than 0.001).

with increased calcification, such as normal growth and osteitis.[12] Alkaline phosphatase decreases in starvation.[8] It is probable that the lambs with spontaneous white muscle disease had a deficient caloric intake due to their inability to nurse properly. The lambs with induced muscular dystrophy were bottle fed and appeared to consume normal quantities of food. Hence, the decreased alkaline phosphatase activity does not reflect inanition.

Acid phosphatase did not change in white muscle disease. The principal diagnostic significance of acid phosphatase is the increase associated with cancer of the prostate in man.[12] No significance can be attached to the lack of change of acid phosphatase in white muscle disease.

A few determinations of true cholinesterase were made in blood serum from control and diseased animals. As has been previously reported, no true cholinesterase could be demonstrated in the blood serum of lambs with or without white muscle disease. All ruminant animals, a few species of fish, and a few species of birds lack blood serum cholinesterase.[2] The reasons for this biochemical anomaly are obscure.

SUMMARY

In white muscle disease in lambs, and in muscular dystrophy induced by feeding cod liver oil, the serum lactic dehydrogenase increased greatly and, in severe cases, the serum alkaline phosphatase decreases to half its normal value. No change was demonstrated in acid phosphatase. True cholinesterase was absent in serum from both normal and diseased animals.

A highly significant linear correlation exists between serum lactic dehydrogenase and glutamic oxalacetic-transaminase in white muscle disease. No correlation could be demonstrated between serum alkaline phosphatase and glutamic-oxalacetic transaminase. The alkaline phosphatase level in lambs with white muscle disease was reduced only in severely affected lambs with very high transaminase levels.

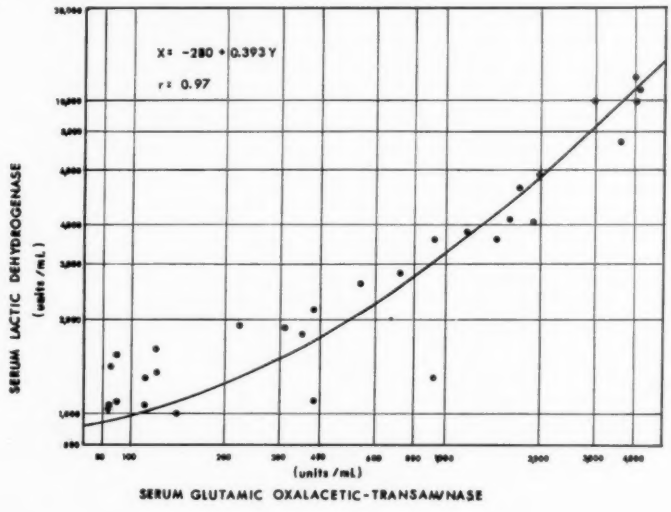

Fig. 1—Interrelationship between serum lactic dehydrogenase and glutamic oxalacetic transaminase in white muscle disease. Logarithmic presentation of data.

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SUMMARIO IN INTERLINGUA

Interrelationes del Enzymas del Sanguine in Morbo a Musculo Blanc (Atrophia Muscular Nutritional)

In morbo a musculo blanc in oves, e etiam in atrophia muscular inducite per dietas de oleo de ficato de gado, le dishydrogenase lactic del sero montava marcatemente. In casos sever, le phosphatase alcalin del sero descendeva al medietate de su nivello normal. Nulle alteration esseva constatate in phosphatase acide. Ver cholinesterase esseva absente in animales normal e in animales malade.

Un significativissime correlation linear existe inter le dishydrogenase lactic e le transaminase glutamic-oxaloacetic in morbo a musculo blanc. Nulle correlation poteva esser demonstrate inter le phosphatase alcalin del sero e le transaminase glutamic-oxaloacetic. Le nivello del phosphatase alcalin in oves con morbo a musculo blanc se monstrava reducite solmente in severmente afficite casos in que le nivellos de transaminase esseva multo alte.

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The Histopathology of Foot-and-Mouth Disease in Pregnant and Lactating Mice

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SUCKLING MICE have been shown to be highly susceptible to foot-and-mouth disease virus (FMDV).[18] Prior to results reported by Campbell,[4] efforts to produce clinical infection in adult mice with the virus were unsuccessful unless some method of virus selection or host treatment was first employed.[6, 10, 15, 16] Campbell[4] found that 40 to 70 per cent of either pregnant mice or lactating mice died after inoculation with FMDV grown in tissue culture. Adult virgin mice, inoculated as controls, were not affected. The purpose of this article is to describe the histopathologic alterations found in pregnant mice and lactating mice that died after inoculation with FMDV.

MATERIALS AND METHODS

The 97th passage of FMDV, type A, strain 119, propagated in bovine primary kidney tissue cultures, was used.[2]

Twenty-seven pregnant mice and 21 lactating mice of the Rockefeller H strain, 90 to 120 days old, were inoculated. The inoculum given to each mouse was 0.1 ml. of undiluted tissue-culture fluid containing about 10^6 plaque-forming units of virus. The virus was injected subcutaneously into the pregnant mice seven days before parturition and intraperitoneally into lactating mice seven or ten days after parturition. Ten pregnant mice died two to four days after the inoculation; 17 survived. Ten nursing mice died one to three days after inoculation; 11 survived. Only the 10 mice that died in each group were included in the histopathologic study.

A total of 27 uninoculated, control mice were killed for histopathologic examination at various intervals during and following gestation; *i.e.*, five, four, and three days before parturition, on the day of parturition, and two, six, nine, ten, and 12 days after parturition. The large number of controls was used to determine normal changes in the thymus in relation to reproduction and, thus, to differentiate physiologic alterations of the thymus from those caused by FMDV infection. The tissues taken for histopathologic examination included thymus, spleen, mesenteric lymph node, myocardium, skeletal muscle, tongue, pancreas, liver, kidney, small intestine, mammary gland, brain, adrenal gland, and fetus tissue. The tissue specimens were fixed in 15 per cent formalin solution, embedded in paraffin, sectioned at 6 μ, and stained with hematoxylin and eosin.

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HISTOPATHOLOGY

Thymus.—Noninfected Mice.—Reported observations[3, 8] that, in some species, the thymus undergoes a temporary physiologic involution during pregnancy made it necessary to determine the possibility of such a phenomenon in mice. An apparently normal histologic pattern of the thymus was seen in 10 noninfected pregnant mice destroyed five, four, or three days before parturition. The wide, dense cortex was packed with small lymphocytes (thymocytes). The central area or medulla contained lesser numbers of small lymphocytes within the reticulum. Thus, the medulla appeared considerably less dense and less darkly stained at low magnification than the cortex (fig. 1). The thymuses in 5 of 7 mice killed on the day of parturition showed a definite departure from the normal histologic pattern. There was appreciable depletion of lymphocytes in the cortical area, resulting in partial loss of the normal dense appearance of the cortex (fig. 2). The thymuses of 2 mice killed two days after parturition showed extreme lymphocytic depletion and, as a result, there was complete loss of differentiation between cortex and medulla under low magnification (fig. 3). Under high magnification, all that was seen in the cortical area was a loose reticulum containing sparsely distributed lymphocytes and lymphoblasts. Presumably, the severe depletion of lymphocytes from the cortex was related to secretion of globulins in the colostrum. In 5 mice examined six days after parturition, there was complete restoration of the normal preparturient histologic pattern of the thymus. The same was seen in mice examined nine, ten, and 12 days after parturition. Thus, it appeared that the thymus in normal mice undergoes a brief physiologic involution within a period be-

Fig. 1—Thymus of normal uninoculated pregnant mouse five days before parturition. The normal cortex is wide and dense. H & E stain; x 27.5

ginning three days before and ending six days after parturition. Subsequently, complete recovery occurred.

Infected Mice.—Histopathologic alterations attributed to foot-and-mouth disease (FMD) were seen in the thymuses of both pregnant and lactating mice that died. A similar pathologic pattern was present in both groups of mice, but the changes developed earlier and became more pronounced in the lactating mice than in the pregnant animals. The pregnant mice that were examined died five to three days before parturition was due, and before the time of physiologic thymic alteration. The lactating mice died during the period when complete recovery from the physiologic involution was apparent in the control animals. Thus, the pathologic alterations occurred either before or after the period during which the physiologic involution associated with reproduction occurs, and at times when control mice had normal histologic patterns of the thymus.

Mice that died one or two days after inoculation showed slight to marked physical breakdown of lymphocytes in the cortex. Lymphocytic nuclear debris was scattered about in the tissue spaces or was engulfed in the cytoplasm of swollen phagocytic reticular cells (fig. 4). In the cortex, depletion of lymphocytes was so extensive as to render it partially to completely indistinguishable as a separate zone under low magnification. There was complete loss of differentiation between cortex and medulla (fig. 5) in mice that died three days after inoculation and only scattered lymphocytes or lymphocytic debris was present in the reticulum of the cortical area. In some mice, there was slight regeneration (proliferation) of lymphocytes in the medulla and, in consequence, the medulla appeared more dense and darkly stained than the depleted cortical area. This reversal of contrast between cortical area and medulla resembled the reversal

Fig. 2—Thymus of normal uninoculated mouse on day of parturition. Depletion of lymphocytes has resulted in partial loss of the normal dense appearance of the cortex. H & E stain; x 27.5

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Fig. 3—Thymus of normal, uninoculated, lactating mouse two days after parturition. Depletion of lymphocytes has resulted in complete loss of differentiation between cortex and medulla. H & E stain; x 27.5

pattern of the thymus described by Hadlow [9] in mice infected with Coxsackie virus. The reversal pattern was well marked in an infected mouse that died four days after inoculation (fig. 6). The changes observed could thus be interpreted as a pathologic (accidental) involution of the thymus resulting in part, if not completely, from destruction of the thymic lymphocytes *in situ*.

Spleen.—The spleens of control mice contained slight to moderate amounts of phagocytized granular basophilic debris in the germinal centers of some of the lymphatic nodules. This was interpreted to be nuclear debris resulting from a low rate of lymphocytic disintegration. However, 2 of the pregnant mice and 9 of the lactating mice that died of FMD had relatively large amounts of nuclear debris in the lymphatic nodules. The debris was not confined to the germinal centers and was seen within the cytoplasm of swollen reticular cells throughout the nodules. The swollen reticular cells containing the phagocytized nuclear debris were sufficiently numerous to give some of the lymphatic nodules a moth-eaten appearance (fig. 7). These changes indicated an increased destruction of lymphocytes and, thus, were basically similar to those observed in the thymus.

Mesenteric Lymph Nodes.—Since lymph nodes react to local as well as to systemic influences, only the mesenteric lymph nodes were included in the study. The pathologic pattern was the same as that observed in the spleen. The mesenteric lymph nodes of control mice contained moderate to large amounts of phagocytized, granular, basophilic material (lymphocytic nuclear debris) in the germinal centers of the lymphatic nodules. Only slight amounts of the granular material were present in occasional reticular cells of the cortex outside the germinal centers. Nine of the lactating mice and 3 of the pregnant mice that died of FMD were shown to have a definite and, in most instances, a well marked increase of the granular basophilic material in the cortex outside the germinal centers. Areas where this material was markedly in-

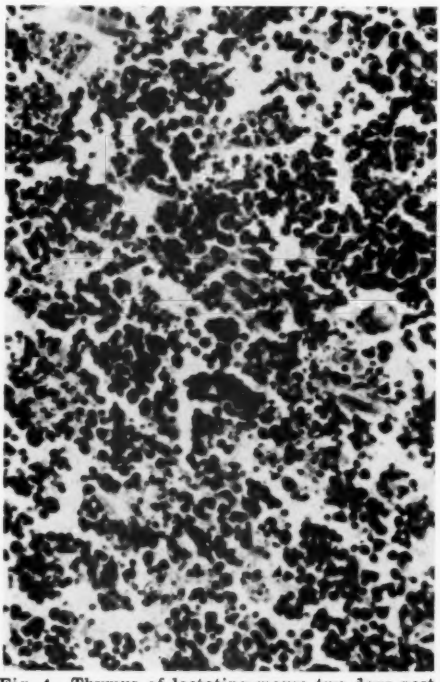

Fig. 4—Thymus of lactating mouse two days post-inoculation (12 days after parturition). There are pyknosis and karyorrhexis of the lymphocytic nuclei. Nuclear debris has been engulfed by reticular phagocytes. H & E stain; x 410

Fig. 5—Thymus of pregnant mouse three days postinoculation (four days before parturition). Destruction and depletion of lymphocytes have resulted in complete loss of differentiation between cortex and medulla. H & E stain; x 27.5

creased had the same moth-eaten appearance noticed in lymphatic nodules of the spleen and were caused by swelling of reticular cells from phagocytosis of the lymphocytic nuclear debris (fig. 8).

Pancreas.—Degeneration and necrosis of the pancreatic acinar cells were seen in every mouse that died of FMD. The amount of epithelial damage varied from moderate to extensive in different animals. Degenerating cells had swollen nuclei with shrunken nucleoli and an increased amount of clear nuclear sap. Many cells contained one or more basophilic, concentrically laminated bodies in the basal portion of the cytoplasm (fig. 9). These bodies ranged in diameter from about 2 to 10 μ and were morphologically similar to the inclusions described by Dineen and Barter [7] in adult mice infected with the PL.5 strain* of pleurodynia virus.[1] Cells that had become necrotic were reduced to anuclear eosinophilic masses. Nuclear changes resembling granular inclusions were seen in a few cells that were undergoing necrosis. There was margination of the chromatin and the nucleus was filled with mauve amphophilic granules (fig. 10). As reported by Platt,[13] the pattern of cellular damage was very

Fig. 6—Thymus of pregnant mouse four days postinoculation (three days before parturition). The "reversal" pattern is well marked. H & E stain; x 27.5

irregular at both the acinar and lobular levels. It appeared that the cells were reacting to the virus individually. In many acini there were both necrotic and degenerating cells; in others, there were both degenerating and relatively normal cells. At the lobular level, completely necrotic acini usually were seen interspersed with partially damaged and normal acini (fig. 11). Changes were not observed in the pancreatic islets.

There also was a moderate to well-marked inflammatory reaction, depending on the amount of epithelial damage. The interlobular connective tissue was distended by inflammatory edema and infiltrated with a

* Virus obtained from throat washings of a patient three days after development of pleurodynia.

XUM

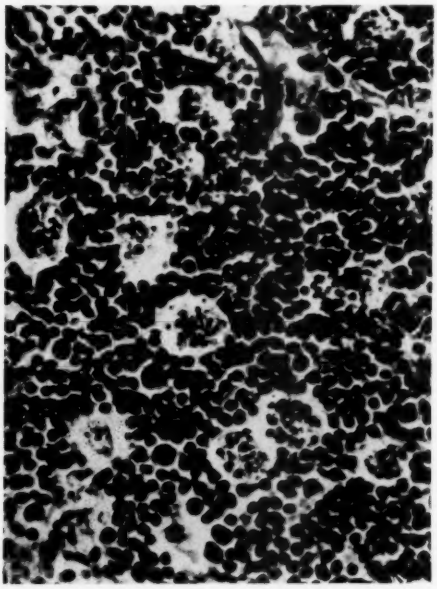

Fig. 7—Lymphatic nodule in spleen of a lactating mouse three days postinoculation (ten days after parturition). Phagocytic reticular cells contain much lymphocytic nuclear debris. H & E stain; x 410.

mixture of granulocytes and monocytes. In mice that lived until the third or fourth day after inoculation, necrotic epithelial debris within the lobules had been invaded and was being phagocytized by large mononuclear cells.

Myocardium.—All mice that died of FMD had appreciable to extensive myocardial degeneration and necrosis. Degenerating muscle fibers contained numerous small vacuoles between the myofibrils. With advancing degeneration, the affected segments of the muscle fibers became swollen and both the longitudinal and cross striations disappeared. At this stage, contraction clots were sometimes present. As necrosis ensued, the nuclei disappeared and the muscle fibers disintegrated into irregular masses of amorphous and granular debris (fig. 12). In mice that died three or four days after inoculation, there also was moderate patchy infiltration of the myocardium with granulocytes and monocytes (fig. 12). The ventricular myocardium was most markedly affected, but some auricular fibers also had undergone degenerative changes.

Skeletal Muscle.—Random sections were made of muscles in the hindlegs, forelegs, thoraces, and abdominal regions of 4 pregnant and 3 lactating mice that died of FMD and of 5 pregnant and 8 lactating control mice. Some damage of the skeletal muscle was seen in all of the infected mice, but less than in the myocardium. In general, the damage was manifested by the presence of a few, isolated, degenerating or necrotic muscle fibers in the different areas examined. Occasionally, small groups of degenerating and necrotic muscle fibers also were seen. However, the diaphragm was markedly damaged and, in some microscopic fields, almost all of the muscle fibers had evidence of pathologic alteration (fig. 13). Degenerating muscle fibers were vacuolated and did not have either longitudinal or cross striations. The sarcolemmic nuclei were pyknotic. Necrotic muscle fibers had undergone disintegration into masses and segments of amorphous eosinophilic debris.

Tongue.—Vesicles were not seen in the lingual mucosae of the mice that died of

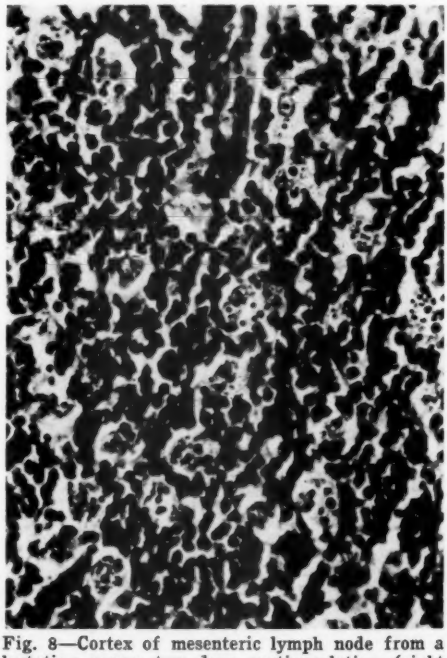

Fig. 8—Cortex of mesenteric lymph node from a lactating mouse two days postinoculation (eight days after parturition). Phagocytic reticular cells contain much lymphocytic nuclear debris. H & E stain; x 410.

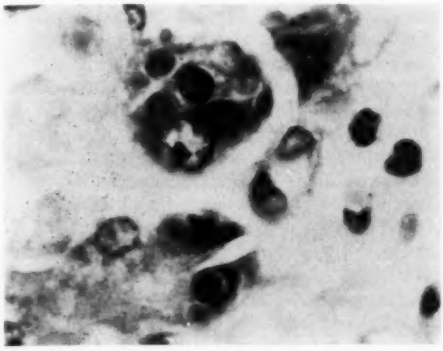

Fig. 9—Pancreas of a lactating mouse two days postinoculation (12 days after parturition). Notice laminated bodies in cytoplasm of pancreatic acinar cells. H & E stain; x 860.

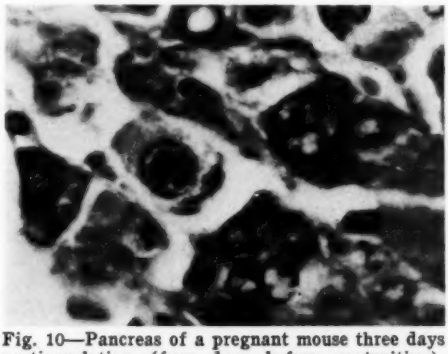

Fig. 10—Pancreas of a pregnant mouse three days postinoculation (four days before parturition). There are nuclear changes in an acinar cell undergoing necrosis. H & E stain; x 860.

FMD. Degenerating and necrotic muscle fibers were seen in the lingual musculature, but greater number were found in the ventral portion of the root of the tongue than elsewhere.

Liver.—Nonspecific changes were found in the livers of mice with lethal infection. There was a variable degree of passive congestion. The hepatic cells were somewhat shrunken and had the compact cytoplasmic structure indicative of glycogen depletion. In many of the cells there were also small cytoplasmic vacuoles characteristic of fatty change. Four of the mice also had slight to well-marked central hepatic necrosis produced by anoxia.

Kidneys.—There was some swelling and vacuolation of the epithelium of the proximal convoluted tubules. These changes were not sufficiently pronounced or consistent to be interpreted as specific effects of the virus.

Small Intestine.—Significant pathologic alterations were not seen in the mucosa.

Lungs.—The lungs were congested. In some instances there was pulmonary edema also.

Brain.—Significant pathologic alterations were not found.

Adrenal Glands.—Special stains were not used to evaluate the status of the adrenal glands. However, in mice that died of FMD, the cytoplasm of the cortical epithelial cells was generally more dense and granular and less spongy in appearance than in the controls. This alteration suggests some depletion of lipids in the adrenal cortex.

Mammary Glands.—Pathologic alterations were not observed in the mammary glands of the pregnant mice that died of FMD. In 7 of the 10 lactating mice that died, there were necrotic, desquamated, epithelial cells in some of the mammary acini.

Fetuses of Pregnant Mice.—Judging from the histologic evidence of postmortem changes, a majority of fetuses that were

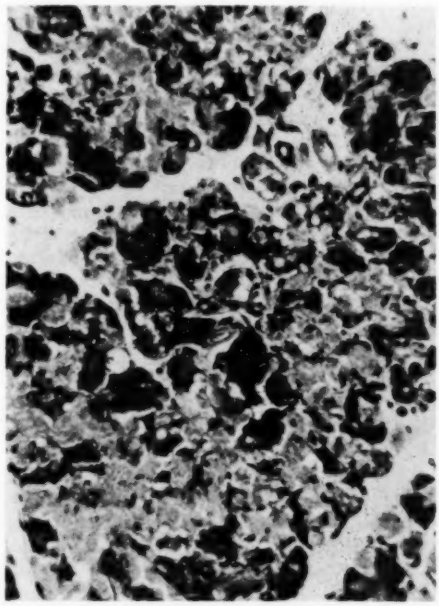

Fig. 11—Pancreas of a pregnant mouse three days postinoculation (four days before parturition), showing pattern of necrosis. H & E stain; x 185.

XUM

Fig. 12—Myocardium of a pregnant mouse three days postinoculation (four days before parturition), showing necrosis of myocardial fibers and cellular infiltration. H & E stain; x 410.

examined had died *in utero*. It was necessary, therefore, to evaluate postmortem changes in relation to possible pathologic alterations in order to draw conclusions. Fetuses of several pregnant mice that died of FMD were shown to have alterations in some of the larger and more developed voluntary muscle fibers that could be interpreted as degenerative changes caused by the virus. The cytoplasm of affected muscle fibers contained fine vacuoles and had an increased affinity for eosin. There was pyknosis or karyorrhexis of the centrally located nuclei. None of the other fetal tissues had noteworthy alterations.

DISCUSSION

The general pattern of pathologic alterations in the pancreas, myocardium, and skeletal muscles of the pregnant and lactating mice was similar to that observed by Platt [13] in 3½- to 4-week-old mice inoculated with the Ven. I strain (Vallée O type) of FMDV. He observed extensive necrosis of the pancreas and myocardium and lesser damage of the skeletal muscles. However, laminated bodies in the cytoplasm of degenerating pancreatic acinar cells, as observed in this study, have been reported only in adult mice infected with PL. 5 pleurodynia virus.[7] Efforts by electron microscopy to demonstrate virus particles within the bodies associated with pleurodynia virus were inconclusive.[14]

Destruction of lymphocytes has been observed in other virus infections. Accidental involution of the thymus with karyoklasis of the cortical lymphocytes was reported by Hadlow [9] in suckling mice infected with Colorado tick fever virus. It was interpreted to be a reflection of stress. No changes in the lymph nodes and spleen were reported. A virus contaminant isolated from leukemic mice by Law and Dunn [11] caused severe lymphocytic depletion of the lymph nodes and spleen. The thymus showed depletion of cortical lymphocytes (accidental involution) and a relative abundance of lymphocytes in the medulla, a reversal of the normal cortico-

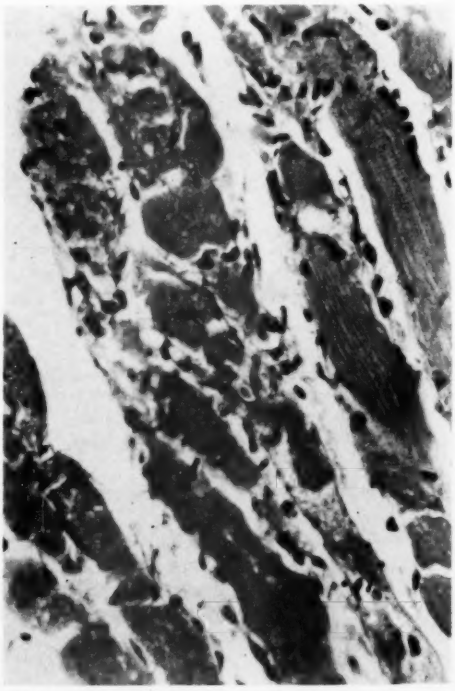

Fig. 13—Diaphragm of a lactating mouse that died of foot-and-mouth disease. Necrosis of voluntary muscle fibers. H & E stain; x 410.

medullary pattern. The picture was similar to that seen in later stages of the "alarm reaction" to nonspecific stress.[17] However, the process (virus infection) apparently affected the lymphocytes and the lymphocytic system selectively. Rinderpest is another disease in which the virus apparently affects the lymphoid cells selectively, although the epithelium of the gastrointestinal tract is also involved.[12] Destruction of lymphatic tissue has been suspected of playing a role in the lethal mechanism of distemper in mink and ferrets.[5]

Thus, when considering the pathogenesis of damage and depletion of lymphocytes in viral diseases, one can postulate both the role of nonspecific stress and the role of a direct interaction of virus and lymphocytes. Lymphocytic destruction from nonspecific stress would be mediated by glucocorticoids liberated from the adrenal cortex.[17] Presumably, a destructive effect of the virus itself upon the lymphocytes could be caused either by multiplication of virus in the cells or by an antigen-antibody reaction in or upon the cell. It is conceivable that a combination of hormonal and viral effects could mediate the lymphocytic destruction seen in some of the viral diseases.

SUMMARY

Histopathologic studies of pregnant and lactating mice infected with type A foot-and-mouth disease virus (tissue culture) revealed that the major pathologic alterations were necrosis of pancreatic acinar cells, development of laminated intracytoplasmic bodies in some of the pancreatic acinar cells, severe myocardial necrosis, slight skeletal muscle necrosis, "accidental" involution of the thymus, and destruction of lymphocytes in the spleen and lymph nodes. The possible pathogenic relationships of both nonspecific stress and direct action of virus to the lymphocytic destruction were discussed.

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SUMMARIO IN INTERLINGUA

Le Histopathologia de Febre Aphthose in Muses Pregnante e Lactante

Studios histopathologic de muses pregnante e lactante, inficite con virus de febre aphthose typo A (ab histoculturas), revelava que le major alterationes pathologic esseva necrose de cellulas acinar pancreatic, disveloppamento de laminate corpores intracytoplasmatic in certes del cellulas acinar pancreatic, sever necrose myocardial, involution "accidental" del thymo, e destruction de lymphocytos in le splen e le nodos lymphatic. Es discutite le possibile relation pathogenetic inter (1) le stress nonspecific e le action directe del virus e (2) le destruction lymphocytic.

The Uterine Biopsy Technique for Following the Histologic Changes Caused by *Vibrio fetus* in the Uterine Mucosa

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Morgantown, West Virginia

MOST STUDIES of the uterine mucosa of cattle have been made using specimens obtained after slaughter. Such samples may not accurately reflect the true histologic structure of the tissue either due to changes resulting from death or because the tissue obtained at that particular time might be atypical. The uterine biopsy technique permits a continual study of the endometrial structure and makes possible a definite evaluation of changes which may occur.

The biopsy technique has been used successfully by Andrews and McKenzie,[1] Miller,[11] Szeky and Dozsa,[16] and Zurgilgen [20] to evaluate changes in the uterine mucosa related to the estrous cycle. No artifacts due to the biopsy technique were found. Skjerven [15] described the cytologic and histochemical changes in the uterine mucosa during the estrous cycle. Brus,[5] Walther *et al.*,[19] Brodauf *et al.*,[4] Grunert,[8] and Schnider [13] found that the biopsy technique is a valuable diagnostic aid, especially in subacute and subclinical cases of endometritis. Hardenbrook [9] believed that additional information obtained from biopsy specimens permitted more appropriate treatment and control measures.

Although there is considerable information on the use of uterine biopsy in cattle, little is known about the effect of specific infectious agents upon the uterine mucosa. The uterine biopsy technique provides a means of evaluating the pathologic effects of an infection upon the endometrium and the duration of the changes resulting from the disease. The latter is of particular interest in terms of the effect upon subsequent reproductive performance.

Vibriosis is a disease of increasing importance and a clear understanding is needed regarding the effects of the disease on the uterine mucosa. This should become a basis for its control and an assessment of its effect upon reproduction. Simon and McNutt [14] described the histologic changes in Vibrio-infected heifers, which were slaughtered at various times following inoculation.

This report is a study of the histologic changes of the uterine mucosa resulting from vibriosis in nonpregnant cows.

MATERIALS AND METHODS

Seven dairy cows, 3 to 6 years of age, were used in this investigation. According to their complete breeding history, these cows were healthy and had calved without difficulty three to four months prior to being used for the experiment. They were housed in isolated pens during the entire experiment. A USDA grade No. 2 mixed hay was fed *ad libitum*.

Two cows served as controls. Only 1 of the control cows was infused with thiol medium when the experimental animals were inoculated. Five cows were inoculated *in utero* with 10 ml. of a 1:4 dilution (thiol medium diluent) of a 72-hour culture of *Vibrio fetus* that had been stored at 4 C. for one to ten days. Motility of the organisms was checked by microscopic examination before each inoculation. The inoculation schedule was as follows: 2 cows were treated during the first 16 hours of heat; 2 in the middle of the estrous cycle, about day 10; and 1 during the last part of the cycle, day 17.

Prior to biopsy, the external genitalia were thoroughly cleaned with soap, water, and disinfectant. Biopsy was accomplished by using a 23-inch long Universal biopsy forceps equipped with a size 1 Krause Universal tip * (fig. 1). This instrument was inserted through the cervix and into the uterus. The uterine area to be biopsied was brought in contact with the forceps by manipulation through the rectum.

Three and eight days before inoculation, cervical mucus samples were taken for culturing and uterine biopsies were performed on all cows. After inoculation with *V. fetus* culture, cervical mucus was collected, and duplicate uterine biopsies were made on each cow every five days during the 120 days of the experiment period. One of these biopsy samples was ground in thiol medium and cultured

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* Manufactured by the United Surgical Supplies Co., Inc., Port Chester, N.Y.

for *V. fetus* using the procedure of Bryner and Frank.[6] All isolations were checked for catalase activity by using the modified catalase test described by Bloom and Christensen.[3] The second biopsy sample was used for histologic examination. Immediately after the sample was obtained it was placed in 10 per cent formalin, prepared by routine histologic methods, and stained with hematoxylin and eosin.

RESULTS

Four of the 5 inoculated cows became infected, as determined by histologic and culture methods employed.

During the 120-day experiment period, it was possible to isolate *V. fetus* from the 4 infected cows. Vibrio was not isolated from the control cows.

The histologic changes in the uterine mucosa of each of the infected cows were similar. These changes will be described for the following periods: five to 30 days, 31 to 60 days, 61 to 90 days, and 91 to 120 days after inoculation with *V. fetus*. The uterine mucosa of the control cows and the *V. fetus*-inoculated cow which did not become infected showed no abnormal histologic changes during the four-month period.

Changes at Five to 30 Days.—The histopathologic changes of the uterine mucosa were evident on the fifth day after inoculation. These changes consisted of moderate disorder and partial detachment of the surface epithelium. A few "stift" cells which could be seen at this time showed the characteristically slender, dark nuclei. The superficial layer of the stroma showed slight, diffuse lymphocytic and mast cell infiltration. However, the deeper layer did not show these changes. The uterine gland pattern was not disturbed, but a few of them contained more than the normal amount of cell debris.

Starting with the 15- to 20-day period, the histologic changes became more pronounced. The surface epithelium was often detached; its upper layer showed marked vacuolization and a darker basophil staining (fig. 2). Every biopsy specimen had a moderate infiltration of the surface epithelium with polymorphonuclear leukocytes and lymphocytes (fig. 3). The superficial layer of the stroma was more dense than normal, and there was moderate polymorphonuclear lymphocyte, mast cell, and plasma cell infiltration. Marked eosinophilia occurred in this layer. The deeper part of the stroma contained moderate numbers of polymorphonuclear leukocytes. A few uterine glands had slight degeneration of the epithelial lining, and a few were partially or completely filled with cell debris. The vessels in the subepithelial layer of the stroma were congested. This did not occur in the deeper layer.

Changes at 31 to 60 Days.—The surface epithelium was markedly disorganized, partially detached, or missing. The vacuolization or shrinking of the cell plasma was considerable, and a few cells had pyknotic nuclei. The neutrophilic infiltration tended to decrease. The subepithelial layer of the stroma contained a large number of neutrophilic granulocytes and leukocytes. Mast and plasma cells were irregularly present. While the neutrophil and lymphocyte infiltration of the compacta was marked and either diffuse or herdlike (focal), that of the spongiosa was not conspicuous (fig. 4). Many glands had markedly degenerated epithelial linings that were rather cuboidal with very indistinct contours. These glands were nearly always filled with degenerated cell fragments. Some dilated gland lumina contained polymorphonuclear cells and lymphocytes (fig. 5). Pronounced hypertrophy of the elastic fibers of many vessels was noticeable.

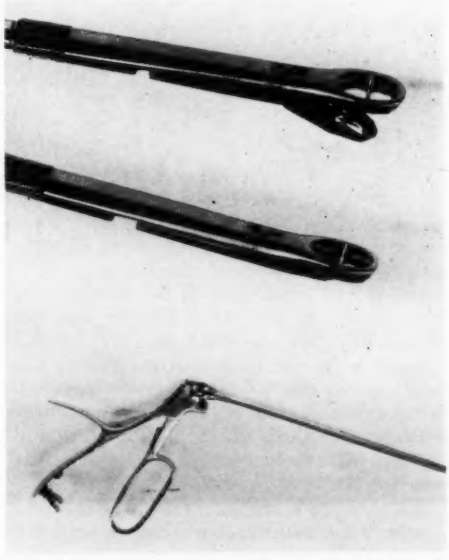

Fig. 1—The upper two pictures show the tip of the biopsy forceps, enlarged three times; the lower picture shows the handle of the instrument, reduced to 1/6 normal size.

XUM

Changes at 61 to 90 Days.—The surface epithelium was still markedly disorganized and degenerated, and in many cases it was completely detached or missing. The surface epithelium of most of the sections had very few inflammatory cells. During this period, a sharp decline of the polymorphonuclear cells occurred in the propria, and the round cells dominated in both layers. The lymphocyte infiltration was focal in most of the sections. Infiltration was seen mostly in the superficial layer of the stroma. There were a few mast and plasma cells present. The epithelia of many glands were considerably degenerated, with the gland lumina being filled with cell debris. A few glands were surrounded by lymphocytes. Many vessels had severe hypertrophy of the elastic fibers and lymphocytic "cuffing" (fig. 6).

Changes at 91 to 120 Days.—Although there were marked individual differences, the surface epithelium did not differ much from that described for the 61- to 90-day period. This was true for the stroma, the glands, and the vessels. The lymphocyte infiltration, which was previously almost exclusively focal, became rather diffuse. In some of the biopsy specimens, the two layers of the stroma were very dense due to the greater number of stroma cells and hypertrophy of the connective tissue fibers. The glands and the vessels were deteriorated as in the previous period, with only minor variations in appearance.

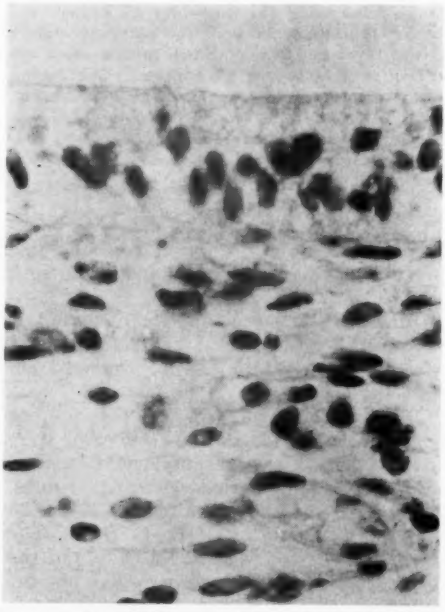

Fig. 2—Vacuolization of the surface epithelium of the uterine mucosa. H & E stain; x 820.

DISCUSSION

There was no alteration of the normal cycle in the cows infected with *V. fetus*. The time of inoculation in relation to stage of estrous cycle had no effect on the severity of the histopathologic changes seen.

One of 2 cows inoculated during the luteal phase of the estrual cycle did not become infected, even though she was inoculated with the same culture and at the same time as the 1 which became infected. This may be another of the exceptions, cited by Black *et al.*,[2] in which high bactericidal activity was shown in some cows in the luteal phase.

The number and distribution of the leukocytes, mast cells, and plasma cells in the endometrium deserve particular attention in estimating the degree of infection present, since their distribution and number are influenced by the severity of the in-

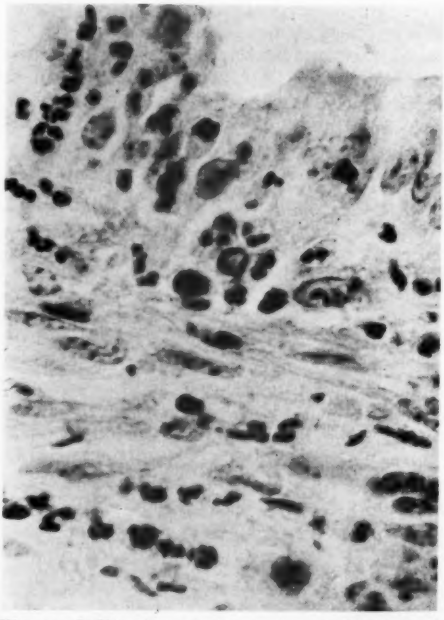

Fig. 3—Infiltration of the surface epithelium of the endometrium with polymorphonuclear leukocytes and lymphocytes. H & E stain; x 820.

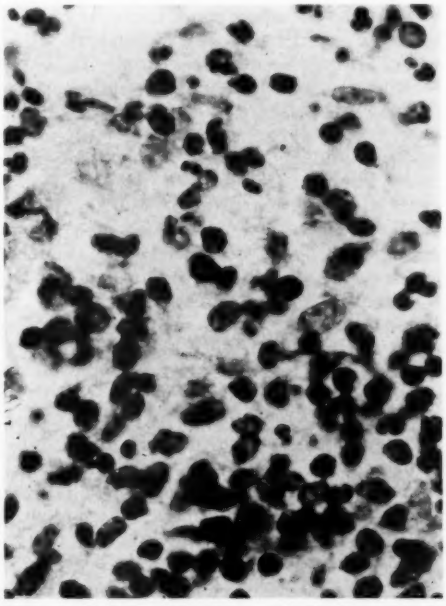

Fig. 4—Polymorphonuclear and lymphocytic infiltration of subepithelial stroma. H & E stain; x 820.

flammatory changes in the uterine mucosa.

In the noninfected control cows, a slight neutrophil infiltration in the subepithelial stroma occurred one or two days before and after estrus. This is in agreement with Skjerven,[15] who stated that the presence of neutrophils in other stages of the estrous cycle should be considered as pathologic. In the cows infected with *V. fetus*, neutrophils were present in all stages of the cycle and were most numerous during the 15- to 60-day period, after which their number decreased. Simon and McNutt[14] found the inflammatory exudate resulting from *V. fetus* infection to be composed of polymorphonuclear leukocytes and lymphocytes.

In the endometriums of healthy cows during the first half of the cycle, lymphocytes were present in small numbers and were usually scattered. The low number of lymphocytes agrees well with earlier observations on slaughterhouse material by Cole.[7] In the Vibrio-infected cows, the lymphocytic infiltration followed a definite pattern. In the first biopsy specimen, taken five days after inoculation, there was only slight diffuse infiltration. Later, during the 15- to 60-day period, they were mixed with polymorphonuclear leukocytes, and the lymphocyte infiltration was diffuse or focal. With the decrease of the neutrophils, the lymphocyte infiltration became primarily focal; whereas, between 91 and 120 days, it was of a rather diffuse nature. The marked and rather uniform lymphocyte infiltration of the uterine mucosa in the infected cows is a sign of chronic endometritis, the severity of the condition being correlated with the degree of infiltration.

The uterine mucosae of the control cows contained only a moderate number of eosinophils, and they were found only just before and after estrus. Moberg[12] did not find any definite cyclic variation in the number of eosinophils, although the greatest number was observed one to two days after estrus. In the Vibrio-infected cows, there was a definite diffuse eosinophil infiltration of the compacta. In a few biopsy specimens this also occurred in the spongiosa, mostly around the glands. This infiltration was not correlated with any phase of the cycle, and after the 50th day only a

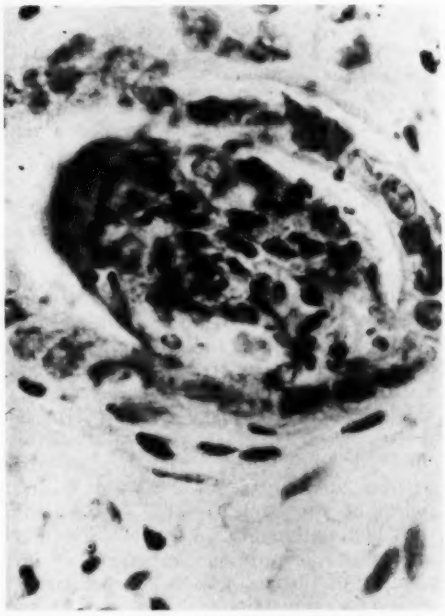

Fig. 5—A partially degenerated uterine gland filled with amorphous cell debris and polymorphonuclear cells. H & E stain; x 820.

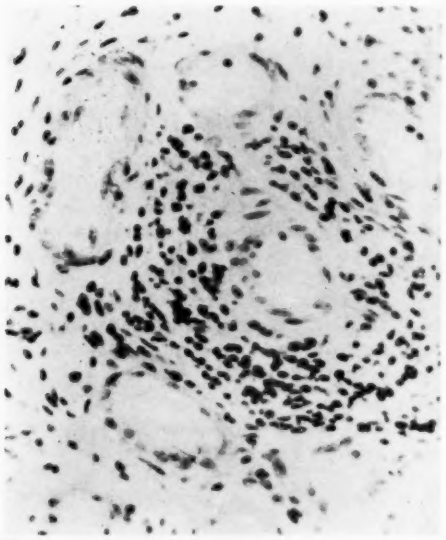

Fig. 6—Lymphocytic "cuffing" around a vessel. H & E stain; x 320.

few scattered eosinophils were found in the stroma. The explanation for their presence in large numbers during the 20- to 50-day period is not apparent.

The presence of varying numbers of mast cells in all stages of the estrous cycle in both controls and infected cows was in agreement with the reports of Simon and McNutt [14] and Skjerven.[15]

The number of plasma cells in the control cows varied markedly. In the Vibrio-infected cows, their number also varied without any apparent correlation with the phase of the cycle or the seriousness of the histopathologic changes. Schnider [13] expressed the opinion that a rather large number of plasma cells is characteristic of chronic endometritis. Szeky and Dozsa [17] concluded that the presence of varying numbers of plasma cells in the stroma is not necessarily a sign of a persistent inflammatory process in multiparous animals.

A feature of the uterine mucosa which is still under debate is the presence of amorphous material in the lumina of uterine glands. Examination of the slides of the uterine mucosa of the control cows showed only a slight amount of cell debris in the otherwise normal gland of the healthy endometrium. Simon and McNutt [14] described many endometrial glands containing amorphous material. In the control cows, some uterine glands occasionally contained a slight amount of cell debris. However, when the surface epithelium, the stroma, and the glands of the endometrium from cows infected with *V. fetus* underwent pathologic changes, the lumina of many glands contained amorphous material. The presence of cell debris in such cases may be regarded abnormal.

With the progression of the tissue changes in the infected cows there occurred a pronounced hypertrophy of the elastic fibers of the vessels of the spongiosa. In the control cows, such changes were only occasionally detected. Whether this hypertrophy is a part of the histopathologic changes of chronic endometritis resulting from *V. fetus* or is a sign of previous pregnancies needs to be defined by further study.

The histologic examination of a uterine biopsy specimen should be of particular value for the diagnosis of subclinical endometritis.

SUMMARY

This report describes a uterine biopsy technique which provided a method of following the course of *Vibrio fetus* infection in the uterine mucosae of cattle. The major changes in the endometrium were slight to marked degeneration of the surface epithelium and moderate to marked polymorphonuclear and lymphocytic infiltration of the stroma. These changes started five days after inoculation, reached a peak between the 30th and the 60th days, and persisted to a lesser degree until the end of the experiment at 120 days. The Vibrio infection did not alter the estrous cycle, nor were the changes influenced by the phase of the cycle at the time of inoculation.

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SUMMARIO IN INTERLINGUA

Le Technica de Biopsia Uterin in le Studio Diachronic del Alterationes Histologic Causate per Vibrio Fetus in le Mucosa Uterin

Le presente reporto describe un technica bioptic que permitte le observation del curso de infection per *Vibrio fetus* in le mucosas uterin de bestial. Le major alterationes in le endometrio esseva leve a marcate grados de degeneration del epithelio superficial e moderate a marcate grados de infiltration polymorphonuclear e lymphocytic del stroma. Iste alterationes se initiava cinque dies post le inoculation. Illos attingeva lor culmine inter le trentesime e le sexantesime die e persisteva minus marcatemente usque al fin del periodo experimental de 120 dies. Le infection vibrional non alterava le cyclo estrual, e le alterationes non se monstrava influentiate per le phase del cyclo al tempore del inoculation.

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Characterization of *Vibrio fetus* Antigens. III. Study of Serologic and Biophysical Properties of a Polysaccharide Fraction in a Hemolytic System

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A phenomenon resulting from the ability of some viruses and bacteria to agglutinate erythrocytes of certain species is generally referred to as direct viral or bacterial hemagglutination. Keogh *et al.*,[6] Alexander *et al.*,[1] Boyden,[2] and Hayes[4] were among the first to demonstrate that certain bacterial antigens may be adsorbed on red blood cells, thus rendering these erythrocytes specifically agglutinable or lysable (in the presence of complement) by the serum antibody directed against the substance adsorbed. Agglutination or hemolysis of red blood cells by antibody against the substance adsorbed is generally referred to as indirect microbial hemagglutination and hemolysis, and has been demonstrated with a number of bacterial antigens.[8]

An immunologically active polysaccharide (HS) fraction has been isolated from smooth *Vibrio fetus* cells of bovine origin.[10] Sheep erythrocytes sensitized with this polysaccharide have been agglutinated by specific antiserums produced in rabbits and by serums of naturally infected cattle.[11] The results of these hemagglutination (HA) tests revealed that the fraction HS of strain UF was antigenically related to a number of individual strains, but did not represent a species-wide antigen.

In this study, sheep erythrocytes sensitized with fraction HS from two strains of *V. fetus* have been employed in a more sensitive hemolytic (HL) test to study serologic cross reactions among various *V. fetus* strains. In addition, some basic serologic and biophysical properties concerning the mechanism of adsorption of the HS fraction by the erythrocytes have been investigated.

Materials and Methods

The methods for producing fraction HS and anti-whole smooth cell serum and for conducting whole cell agglutination tests (AG) have been described.[11, 12]

Reagents Used in the Hemolytic Test.—Sheep erythrocytes were collected, stored, diluted, and sensitized as in the HA test,[11] except that they were washed immediately before sensitization and the 2.5 per cent erythrocyte suspension was standardized to contain approximately 380 x 10^6 cells per cubic millimeter. Unless otherwise indicated, the minimal sensitizing dose (m.s.d.), *i.e.*, the smallest amount giving maximum titer with homologous serum, was used in sensitization. The m.s.d. for the HS fraction of strain 3849 was 160 μg. per milliliter of 0.5 per cent erythrocyte suspension, and 10 μg. per milliliter for the HS fraction of strain UF.

Serums were inactivated in a water bath at 56 C. for 30 minutes. Normal sheep erythrocyte antibody was removed from the serums to be tested by absorbing twice at 37 C. for 10 minutes with washed, packed, normal sheep erythrocytes totaling 1/10 the total serum volume.[11] Thimerosal* was added to make a final dilution of 1/10,000 and the serums were stored at 4 C.

Pooled serums from approximately 20 male guinea pigs were the source of complement. Four hours after collection, serum was removed from the whole blood. It was pooled and stored in sealed ampules under dry ice refrigeration, and was thawed and diluted in cold saline solution immediately before use.

Performance of the Hemolytic Test.—Serial twofold dilutions of the antiserum to be tested were prepared in 0.85 per cent NaCl solution to give final volumes of 0.5 ml. To each serum dilution tube, 0.1 ml. of a 1:10 complement was added, followed by 0.5 ml. of 0.5 per cent sensitized cell suspension per tube. To ascertain eventual hemolytic activity of complement, serum, and saline solution, three control tubes were incorporated in the tests. The tubes were shaken and incubated at 37 C. for one hour, when final readings were made. The hemolytic titer of the serum was considered

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* Merthiolate, produced by Eli Lilly & Co., Indianapolis, Ind.

to be the highest dilution of serum with complete hemolysis. Partial hemolysis was indicated by presence of a red lysate and a sediment of apparently intact erythrocytes. The fluid reacting negatively contained apparently intact erythrocytes.

Measuring Quantity of HS Actually Adsorbed by Erythrocytes.—The quantity of HS substance actually adsorbed to a given quantity of erythrocytes was determined indirectly by measuring the quantity of fraction remaining free in the supernatant fluid after sensitization. The supernatant fluids of three washings of the sensitized cells were pooled, diluted when necessary, and then used to sensitize additional erythrocytes under conditions identical with those of the initial sensitization. These erythrocytes were then permitted to react with an already standardized serum. On the basis of titers obtained in this test, it was possible to calculate the quantity of the HS fraction still free in the supernatant fluid. The quantity of the HS substance actually absorbed in the initial sensitization was calculated from the data in table 2.

Multiple Sensitization of Erythrocytes.—The capacity of erythrocytes to adsorb two antigenically heterologous HS antigens was investigated by simultaneous, consecutive, and saturation treatment of erythrocytes with these antigens. The latter attempt was aimed at determining whether the adsorbing surface of the erythrocytes could be saturated with one HS substance to the extent that it would interfere or block the subsequent adsorption of the heterologous HS antigen.

Specificity of the Hemolytic Test—Specificity of the HL test for *V. fetus* with reference to Brucella and Leptospira species was studied. Rabbit antiserums directed against formalin-killed *Brucella abortus* strain BAT 1119-3; *Leptospira pomona* strains LC5–71, LT–91, S–91; *L. canicola; Leptospira icterohemorrhagiae;* and *Leptospira sejroe* were used in the hemolytic system with two erythrocyte suspensions sensitized with antigens UF and 3849, respectively.

RESULTS

Results of the HL and AG tests with 21 *V. fetus* antiserums are shown (table 1). With few exceptions, some degree of cross-reactivity among antigens UF and 3849 and the 21 antiserums was demonstrated. There was no cross reaction between antigens UF and 3849 and their antiserums on the basis of HL tests; however, cross reactions between these strains occurred in AG tests. Serums against bovine-abortive strains B4 and 426 reacted with UF antigen in the HL test but not in the AG test. Similar reactions were observed with antiserums against two strains of human origin. Antiserum against strain DL (isolated from an apparently

TABLE 1—Hemolysis and Whole Cell Agglutination of Two Antigens and Serums Against Smooth Forms of 21 Vibrio fetus Strains

Serum prepared against strain:	Strain UF			Strain 3849		
	AG titer	HL titer	HL titer / AG titer	AG titer	HL titer	HL titer / AG titer
BOVINE ABORTIVE STRAINS						
F	2,560*	10,240	4	1,280	0	>1
UF	5,120	40,960	4	160	0	>1
B1	0	0	....	320	640	2
B4	0	160	160	640	2,560	4
B9	0	0	....	40	2,560	64
B10	640	5,120	8	160	160	1
B12	0	0	....	10,240	5,120	>1
B14	0	0	....	320	5,120	16
B15	20	640	32	1,280	5,120	4
B16	40	640	16	320	5,120	16
426	0	640	640	160	5,120	32
RL5	160	2,560	16	2,560	5,120	2
D71	640	640	1	1,280	20,480	16
3849	640	0	>1	5,120	20,480	4
OVINE ABORTIVE STRAINS						
R3	0	640	640	160	2,560	16
543	2,560	10,240	4	160	160	1
K	5,120	20,480	4	640	0	>1
Ill.	5,120	40,960	8	0	0	.....
BOVINE NONABORTIVE STRAIN						
DL	0	160	160	0	160	160
HUMAN IDIOPATHIC STRAINS						
1154	0	0	....	40	320	8
1133	0	0	....	0	40	40

* Titer expressed as reciprocal of serum dilution. Serums were prepared against whole *V. fetus* cells.
>1 = Ratio titer less than 1.

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TABLE 2—Lysis of Sheep Erythrocytes by a Reference Homologous Antiserum after Sensitization with Polysaccharide (HS) Fraction of Vibrio fetus Strain UF

μg. HS/ml. 0.5% RBC suspension	Serum dilutions											
	40*	80	160	320	640	1,280	2,560	5,120	10,240	20,480	40,960	81,920
20.0	+	+	+	+	+	+	+	+	+	+	+	−
15.0	+	+	+	+	+	+	+	+	+	+	+	−
10.0	+	+	+	+	+	+	+	+	+	+	+	−
5.0	+	+	+	+	+	+	+	+	+	+	−	−
4.0	+	+	+	+	+	+	+	+	+	−	−	−
3.0	+	+	+	+	+	+	+	+	−	−	−	−
2.0	+	+	+	+	+	+	+	+	−	−	−	−
1.0	+	+	+	+	+	+	+	−	−	−	−	−
0.8	+	+	+	+	+	+	+	−	−	−	−	−
0.6	+	+	+	+	+	+	−	−	−	−	−	−
0.4	+	+	+	−	−	−	−	−	−	−	−	−
0.2	+	+	−	−	−	−	−	−	−	−	−	−
0.1	−	−	−	−	−	−	−	−	−	−	−	−

+ = lysis; − = no lysis. * Titer expressed as reciprocal of serum dilution. Antiserums were prepared against whole *V. fetus* cells.

normal bovine animal) reacted with the two antigens in the HL test but not in the AG test. In 27 tests, HL titers were considerably higher than AG titers; in three instances these were equal, and in five tests AG titers exceeded HL titers.

The minimal quantity of the fraction HS required to sensitize 1.0 ml. of a 0.5 per cent suspension of sheep erythroyctes was 10 μg., so that hemolysis with homologous serum prepared against the whole Vibrio cells occurred at maximal titer (table 2). The use of sensitizing quantities of the fraction exeeeding 10 μg. resulted in no increase of the HL titer. The minimum quantity of the fraction still having HL reaction, although in considerably lower serum dilution, was 0.6 μg. of fraction HS per milliliter.

Upon determining of the actual quantities of HS fraction adsorbed, as increasing doses were used to sensitize fixed quantities of the erythrocytes, it was seen that a constant increase in uptake of the fraction by the erythrocytes took place up to the sensitizing dose of 3,200 μg. (fig. 1). Use of sensitizing quantities of HS fraction exceeding 3,200 μg. resulted in no increase of the fraction actually adsorbed by the erythrocytes. However, erythrocytes sensitized in twice this saturation dose (6,400 μg. per ml.) and subsequently sensitized with an m.s.d. of a heterologous HS fraction (1134) were hemolyzed to the same titer obtained when the cells were sensitized only with 1134 of UF HS fraction (table 3).

The HL results of testing erythrocytes sensitized simultaneously with HS antigens

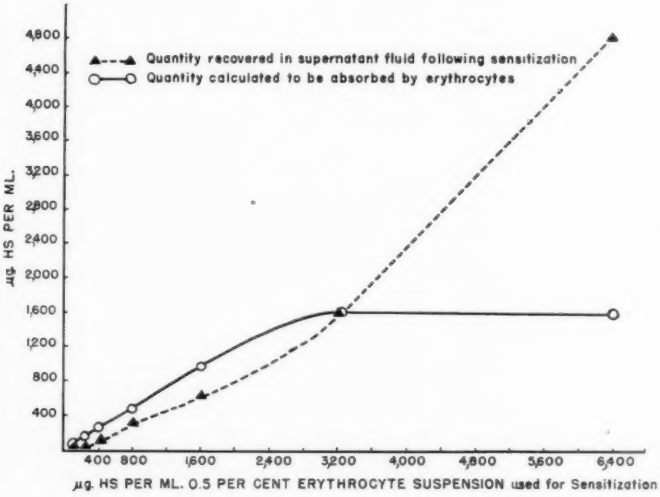

Fig. 1—Relationship between quantities of polysaccharide (HS) fraction of Vibrio fetus cells used to sensitize erythrocytes and quantities actually adsorbed.

TABLE 3—Capacity of Erythrocytes, When Saturated with One HS Antigen, to Adsorb an Antigenically Heterologous Polysaccharide (HS) Antigen

HS antigen	Sensitizing dose	Previous treatment of erythrocytes	Antiserum directed against antigen	HL titer
1134	m.s.d.*	untreated	1134	1:40,000
UF	saturation**	untreated	1134	0
UF	saturation	untreated	UF	1:80,000
1134	m.s.d.	untreated	UF	0
1134	m.s.d.	saturated with UF	UF	1:80,000
1134	m.s.d.	saturated with UF	1134	1:20,000

Antiserums were prepared against whole *V. fetus* cells.
* Minimal sensitizing dose. ** Two times the dose at which no further adsorption was noticed.

UF and B14 are shown (table 4). Cells treated with one antigen reacted only with homologous antiserum, while cells treated with two antigens were lysed by both serums. Lysis of erythrocytes sensitized with two antigens occurred at serum dilutions identical to those in which erythrocytes sensitized with one antigen were lysed.

It is evident that the adsorption of UF HS antigen does not affect the ability of erythrocytes to adsorb, simultaneously or consecutively, another serologically heterologous antigen (table 5). Cells sensitized consecutively by the two HS antigens, UF and B14, were lysed by the antiserums UF and B14 in dilutions 1:10,240 and 1:1,280, respectively. These serum titers were identical or closely corresponded to those where cells treated with one antigen were lysed by their homologous antiserum. When mixtures of erythrocytes, consisting of 50 per cent cells treated with antigen UF and 50 per cent cells treated with antigen B14, were reacted with either serum UF or B14, incomplete hemolysis occurred in all serial serum dilutions ranging from 1:160 to 1:10,000. Similar results were obtained when a mixture comprised of 50 per cent UF-treated cells and 50 per cent nontreated cells were reacted with UF antiserum.

Specific serums prepared against B. *abortus* and three *L. pomona* strains, *L. canicola*, *L. icterohemorrhagiae*, and *L. sejroe* did not lyse the erythrocytes sensitized with HS fractions of *V. fetus* strains UF and 3849 in dilutions of 1:10 and higher.

DISCUSSION

Results of cross-hemolysis reactions among 21 *V. fetus* strains of various origin revealed the presence of at least two serologically heterologous antigens possessed by UF and 3849 strains. Cross reaction between the latter two antigens in the AG test points to the existence of common superficial antigens. Cross reactivity of HS antigens from UF and 3849, H_2S-negative strains, and serum against H_2S-positive strain DL indicates an antigenic similarity of the organisms.[3]

Simultaneous adsorption by erythrocytes of two heterologous HS antigens, UF and B14, resulted in a test antigen with broader serologic activity. This was evidenced by the fact that erythrocytes treated with these two antigens were lysed by each of the serums. This finding suggests that both antigens were adsorbed nonselectively by all erythrocytes. Further proof in support of this statement is that a mixture of equal quantities of UF- and B14-treated cells was only partially reactive in the hemolytic system against the serum of each of these antigens. Both antiserums produced partial lysis of this mixture, lysing completely only the erythrocytes treated with homologous antigen.

Consecutive treatment of erythrocytes with two antigens and an attempt to satu-

TABLE 4—Lysis, by Their Specific Antiserums, of Erythrocytes Sensitized Singly or Simultaneously with Two Heterologous Polysaccharide (HS) Antigens

Dilution of *Vibrio fetus* antiserum		HS antigens used to treat erythrocytes		
		UF	B14	UF and B14
UF	1:160	+	−	+
	1:320	+	−	+
	1:640	+	−	+
	1:1,280	+	−	+
	1:2,560	+	−	+
	1:5,120	+	−	+
	1:10,240	+	−	±
B14	1:160	−	+	+
	1:320	−	+	+
	1:640	−	+	+
	1:1,280	−	+	+
	1:2,560	−	+	±
	1:5,120	−	±	−
	1:10,240	−	−	−

+ = complete lysis; ± = partial lysis; − = no lysis.
Antiserums were prepared against whole *V. fetus* cells.

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TABLE 5—Influence of Consecutive Treatment of Erythrocytes with Two Heterologous Polysaccharide (HS) Antigens Upon Adsorption of These Antigens as Determined by the HL Test

Dilution of *Vibrio fetus* antiserum		HS antigen used for treatment of erythrocytes					
		UF	B14	UF followed by B14	B14 followed by UF	Mixture: 50% UF and 50% B14	Mixture: 50% UF and 50% untreated cells
UF	1:160	+	−	+	+	±	±
	1:320	+	−	+	+	±	±
	1:640	+	−	+	+	±	±
	1:1,280	+	−	+	+	±	±
	1:2,560	+	−	+	+	±	±
	1:5,120	+	−	+	+	±	±
	1:10,240	+	−	+	+	±	±
B14	1:160	−	+	+	+	±	−
	1:320	−	+	+	+	±	−
	1:640	−	+	+	+	±	−
	1:1,280	−	+	+	+	±	−
	1:2,560	−	+	±	±	±	−
	1:5,120	−	±	−	−	−	−
	1:10,240	−	−	−	−	−	−

+ = complete lysis; ± = partial lysis; − = no lysis. Serums were prepared against whole *V. fetus* cells.

rate erythrocyte surfaces with a single HS substance had no effect on the hemolytic titer of any of the systems studied. It is conceivable that blocked adsorption of one antigen may result from previous single or multiple treatments of the cells with another antigen. However, this was not observed. It seems likely that the surface area of the erythrocytes was only partially coated by even the most concentrated polysaccharide solution used in these experiments, thus allowing the adsorption of further polysaccharides. It is understood that the stereochemical configuration of the polar groups of the molecules comprising an antigen is partially responsible for its immunoserologic specificity. With this in mind, one may assume that chemical interaction between certain specific receptor groups of the erythrocyte surface and corresponding linkage of the polysaccharide molecule are determinative in the indirect microbial hemolysis reaction. Landy [7] and Neter,[9] who made similar observations with other bacterial antigens, suggested that different areas of the erythrocytes may be involved for each antigen.

In most tests described, the antiserums produced hemolysis of sheep erythrocytes treated with the homologous antigens at titers exceeding, by far, those causing agglutination of these cells. Heidelberger *et al.*[5] calculated that only 0.3 per cent of the surface of the erythrocyte need be coated with specific antibody to bring about hemolysis in the presence of complement. The hemolytic system described previously differs from the conventional hemolytic system of a complement-fixation test in that the antigen is not the erythrocyte itself but the polysaccharide which is attached to the erythrocyte surface. The combination of antigen with specific antibody in the presence of complement causes lysis of the erythrocytes. The fact that only small amounts of antibody are required for hemolysis in the presence of excess complement explains the sensitivity of the hemolytic test. This indicates that the hemolytic test offers a method for detecting small concentrations of antibody and antigen. Naturally occurring *V. fetus* infection is known to induce a more local than general immunologic response of the host. Under these circumstances, one might expect to detect relatively low concentrations of the circulating antibodies, thus indicating the need of a more sensitive test in the field of diagnostic serology. Investigation of the use of the described HL test for detection of *V. fetus* antibodies and antigens in bovine and ovine infections appears appropriate.

SUMMARY

In this report, a hemolysis test for detecting antibodies against *Vibrio fetus* is described. Sheep erythrocytes sensitized with a polysaccharide (HS) fraction of two *V. fetus* strains were lysed by specific serums produced in rabbits against 21 other Vibrio strains of bovine, ovine, and human origin.

The hemolytic test was found to be superior to the tube-agglutination test in its ability to detect smaller quantities of antibody. Treatment of sheep erythrocytes with

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one of the antigens did not block the simultaneous or subsequent adsorption of the second antigen.

For a series of increasing sensitizing doses of the HS fraction, actual quantities adsorbed by the erythrocytes were determined.

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SUMMARIO IN INTERLINGUA

Characterisation de Antigenos ab Vibrio fetus. III. Studio del Proprietates Serologic e Biophysic de un Fraction Polysaccharidic in un Systema Hemolytic

Es describite un test de hemolyse pro le detection de anticorpore anti *Vibrio fetus*. Erythrocytos ovin, sensibilisate per medio de un fraction polysaccharidic ab duo racias de *V. fetus*, esseva lysate per specific seros producite in conilios contra 21 altere racias vibrional de origine bovin, ovin, e human.

Le test de hemolyse se provava como superior al test de agglutination in tubos per su capacitate de deteger plus micre quantitates de anticorpore. Le tractamento de erythrocytos ovin con un del antigenos non blocava le simultanee o le subsequente adsorption del secunde.

Le quantitate de sensibilisante fraction polysaccharidic de facto adsorbite per le erythrocytos esseva determinate pro un serie de progressivemente plus alte doses de ille fraction.

Studies of Anaplasmosis. I. Filtration of the Causative Agent

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THEILER demonstrated that the etiologic agent of anaplasmosis, apparently contained in the lysate of infected erythrocytes, did not pass through ordinary bacterial filters.[10] Subsequently, a number of investigators conducted studies on the filterability of the causative agent of anaplasmosis in an attempt to ascertain its biologic nature.[1-5] With the exception of Foote,[5] who claims that the ultrafiltrate of the blood from animals with acute anaplasmosis is infective, the above investigators have confirmed Theiler's findings. Ristic[9] has described the structural configuration of three principal *Anaplasma marginale* growth forms and considers the initial-body form (0.3 to 0.4 μ in diameter) to be responsible for initiating and perpetuating the infection.

The purpose of the present study was to show that initial bodies of *A. marginale* could be filtered, thus demonstrating that under the appropriate conditions the causative agent of anaplasmosis is capable of surviving and retaining infectivity in the cell-free state.

MATERIALS AND METHODS

Blood containing 40 to 60 per cent *A. marginale*-infected erythrocytes was taken from cattle with acute anaplasmosis. It was subjected either to mechanical disintegration or lysis by an autoantibody-complement system[8] to liberate the Anaplasma bodies.

Mechanical Disintegration.—Fresh heparinized whole blood containing *A. marginale* (0.5 mg. heparin sodium for 1 ml. of blood) was centrifuged for ten minutes at 3,000 r.p.m. in a centrifuge with a 12-inch diameter head. The packed cells were disintegrated in an homogenizer* at its maximal speed for five minutes at 4 C. Stromata were separated by centrifugation at 4,800 r.p.m. in the described centrifuge for ten minutes. The supernatant lysate was admixed with twice its volume of serum from an anaplasmosis-free animal. The mixture then was filtered through a 0.65 μ (average pore size) Millipore filter,** using 10 lb. of air pressure, into a flask containing 10 ml. of freshly collected heparinized whole blood from an anaplasmosis-free animal. Immediately after filtration, 1 ml. of the Anaplasma-free blood to which the filtrate from infected cells had been added was removed for electron microscopic examination. The remainder was divided into equal volumes and injected subcutaneously and intravenously into the same Anaplasma-free cattle from which blood originally had been obtained. The time required for the entire procedure, including collecting, filtering, and inoculating the specimens, did not exceed one hour.

Lysis by Autoantibody-Complement System.—An autoantibody directed, at least in part, against the erythrocytes of an animal acutely infected with *A. marginale*[8] also was used to liberate Anaplasma bodies from the erythrocytes. The presence of autoantibody on erythrocytes of the infected calf was first ascertained by use of a lytic system consisting of 2 drops of rabbit complement and 2 drops (approximately 0.05 ml. per drop) of a 2 per cent suspension of washed bovine erythrocytes in 0.85 per cent sodium chloride solution. The degree of lysis was determined after incubation of the system for four hours at 20 to 24 C. After it had been ascertained that sufficient autoantibody was present, as indicated by complete lysis with no intact erythrocytes left, 10 ml. of infective whole blood was collected from an animal into an equal volume of Alsever's solution. The erythrocytes were immediately washed in sodium chloride solution and, to 2 ml. of packed erythrocytes, 5 ml. of sodium chloride solution and 2 ml. of rabbit complement were added. The system then was incubated for one hour at 24 C. to permit lysis. The stromata were removed and the supernatant lysate filtered into Anaplasma-free blood as previously described. The quantities of blood used for inoculum and electron microscopic examination and inoculation were similar to that described for mechanical disintegration. Electron microscopy, employing the Philips Model 100 electron microscope with 60 kv., was used to study the organism in its extra- and intra-erythrocytic state.[9] Processing of specimens for electron microscopic examinations were made within one hour following filtration.

The complement-fixation technique of Gates *et al.*[6] was conducted every third day on blood serums of inoculated animals; the conventional Giemsa staining technique was used to detect marginal

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* Virtis "45" homogenizer, manufactured by Virtis Company, Inc., Gardiner, N.Y.

** Millipore filter, manufactured by Millipore Filter Corporation, Watertown, Mass.

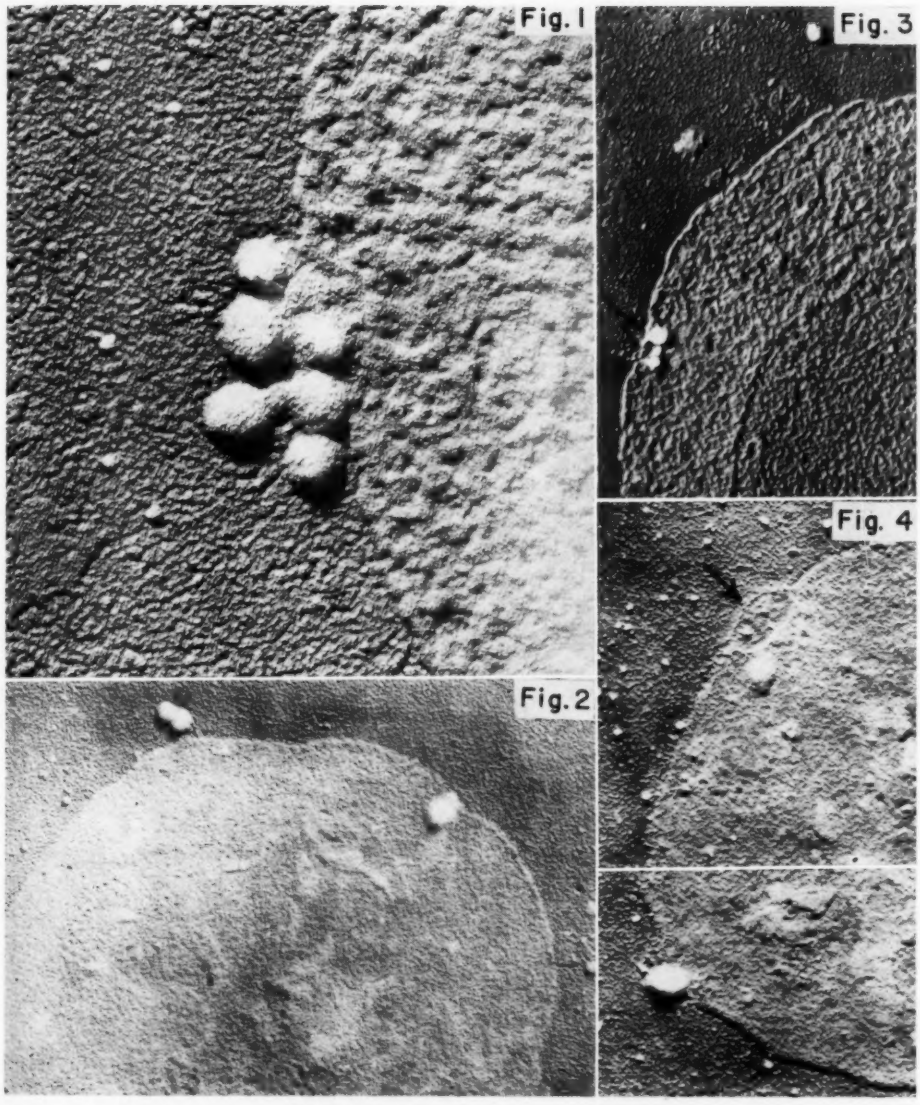

Fig. 1—A group of initial bodies on the erythrocytic membrane. x 32,200.

Fig. 2—A single initial body which may be in the process of penetrating a susceptible erythrocyte. x 11,270.

Fig. 3—Three initial bodies inside the erythrocytic membrane. Notice a fine perforation of the cell membrane. x 7,245.

Fig. 4.—Two initial bodies in the process of penetration of a susceptible erythrocyte. Notice the ruptured erythrocytic membrane in close proximity to the upper initial body. x 12,075.

Anaplasma bodies within the erythrocytes of these animals. The experimental animals consisted of 3 serologically and hematologically negative Guernsey calves (7 to 9 months old) which had been raised in isolation.

Results

Electron microscopic examinations of the blood from Anaplasma-free animals, admixed with the filtrate containing liber-

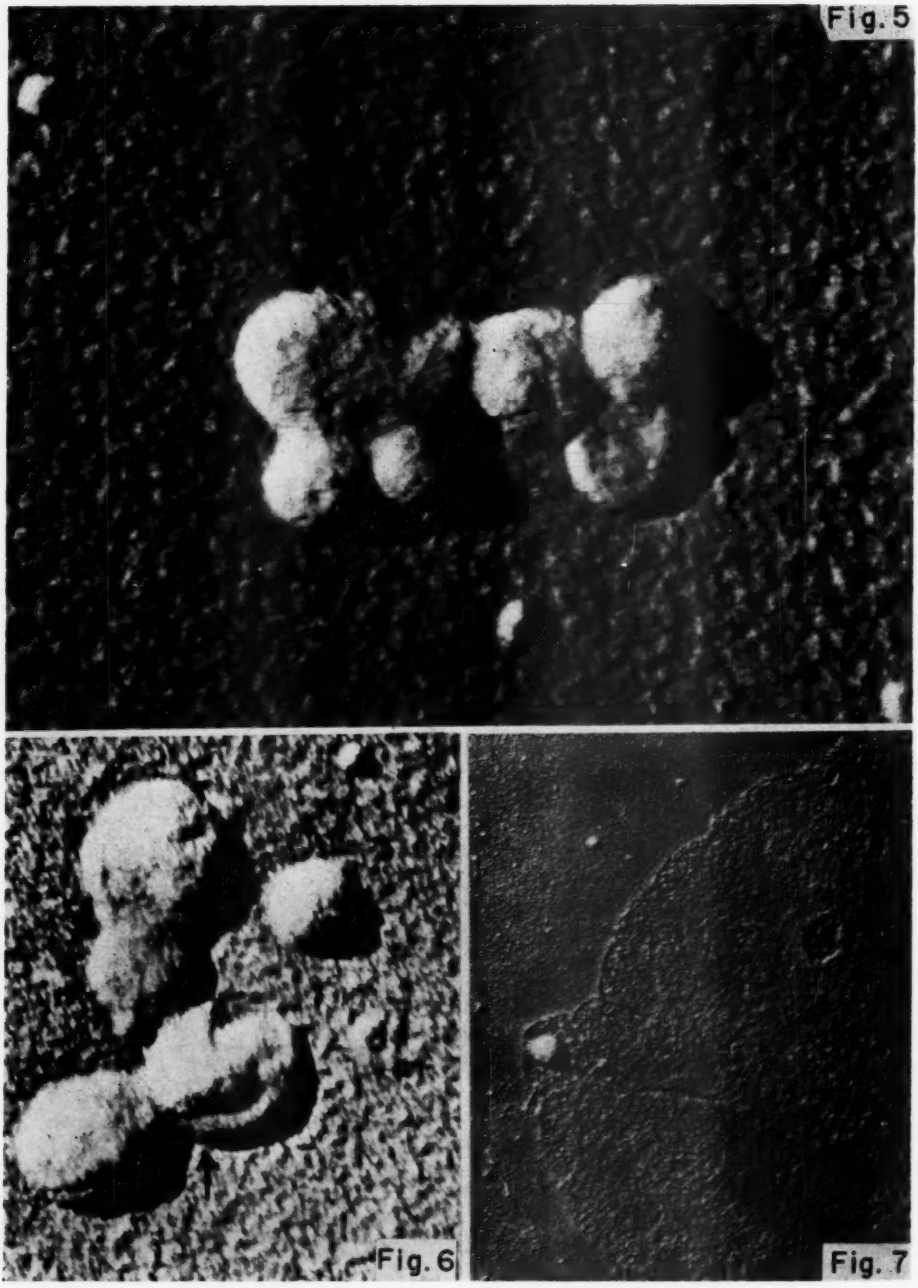

Fig. 5—Initial and polyhedral bodies in the cell-free state. x 36,670.

Fig. 6—Initial bodies occurring in pairs and singly in the cell-free state. A double membrane of the initial body can be observed in at least one of these bodies. x 36,670.

Fig. 7—An initial body in the process of apparent penetration of a susceptible erythrocyte. x 14,670.

XUM

ated Anaplasma material of acutely infected animals, revealed "initial" bodies attached to erythrocytes and cell-free initial and polyhedral bodies. Initial bodies were observed in groups attached upon and inside erythrocytic membranes (fig. 1, 3) and as single forms apparently in the process of penetrating susceptible erythrocytes (fig. 2, 4, 7). The majority of initial and polyhedral bodies occurring in the cell-free state apparently were intact and were dispersed as aggregates (fig. 5, 6). Some of these initial bodies appeared to be damaged. Others, in process of disintegration, were surrounded by polyhedral bodies (fig. 5). A double membrane of the initial body was clearly observed in at least one of these aggregated bodies (fig. 6). Generally, there were fewer free or attached Anaplasma bodies in the filtrate, liberated by the action of the autoantibody-complement system, than were present in the filtrate obtained by mechanical disintegration of infective erythrocytes.

Examination of the lysate of infective erythrocytes by means of the light and electron microscopy before and after filtration revealed that it was free of any intact cellular elements, including erythrocytes.

Inoculations of the 3 Anaplasma-free cattle with their homologous blood specimens, admixed with erythrocytic filtrates of the infected animal, resulted in development of acute anaplasmosis in each animal. Study of 2 animals, inoculated with filtrate secured following mechanical disintegration of the infective erythrocytes, revealed a maximum of marginal Anaplasma bodies in 25 and 30 per cent of their red blood cells at 23 and 27 days, respectively, following inoculation. On these dates, both animals had complement-fixing titers of 1:160. The calf inoculated with the Anaplasma filtrate resulting from autoantibody-complement activity upon infective erythrocytes developed a relatively mild infection with a maximum of 3 per cent erythrocytes containing marginal bodies and a 1:80 complement-fixing titer on the 35th day.

DISCUSSION

Reported evidence, already cited, indicates that the etiologic agent of anaplasmosis does not pass through ordinary bacterial filters. It may be, therefore, that this organism is of appreciable size. Electron microscopic studies of carrier-animal blood revealed the presence of Anaplasma initial within the erythrocytes.[9] This fact accounts for the infectivity of carrier blood upon its inoculation into a susceptible host. Filtration of blood from the acutely infected animal through a filter with an average pore diameter of 0.65 μ, approximately double the size of the initial body, permits its undisturbed passage through the filter.

Fresh whole blood proved to be a suitable substrate for acceptance and preservation of the infectivity of initial bodies. Although additional studies will be required to determine the specific forces governing the survival of initial bodies in the cell-free state, the high sensitivity of *A. marginale* to various salts contained in artificial substrates has been demonstrated.[7] Supporting this observation was the smaller number of initial bodies observed upon susceptible erythrocytes when the method of liberation consisted of the hemolytic system, requiring prolonged maintenance of the organism in a substrate containing sodium chloride. Apparent influence of this treatment upon virulence of liberated Anaplasma bodies could have resulted in the low-grade infection, as measured by the percentage of marginal bodies, in comparison to more severe infections contracted by animals inoculated with the filtrate liberated in a medium free of artificial additives.

A further point of interest is the ability of liberated initial bodies to attach and apparently penetrate susceptible erythrocytes *in vitro*. This observation is of dual significance: (1) it directly indicates the feasibility of further exploring the phenomenon for the purpose of *in vitro* propagation of *A. marginale* to at least the initial-body stage, and (2) it indirectly suggests the existence of a mechanism within the blood vascular system necessary for the liberation of the initial body stage, thus making possible perpetuation of the infectious agent. This mechanism may involve joined forces of the organism and the host within the circulation. Evidence of localized perforation of the membrane of erythrocytes in close proximity to initial bodies in their extra-erythrocytic stage[9] may indicate production of erythrocyte wall-destroying enzymes by the Anaplasma. However, cellular disintegration and phagocytic activity induced by the autoantibody[8] constitute other means by which the initial bodies could be liberated. In addition, the dynamic blood flow could constitute an

indispensable mechanical force, enabling the invading organism to attach rapidly upon susceptible cells.

The techniques described for the liberation and filtration of the initial and polyhedral bodies may be utilized in the study of biophysical and physiologic factors influencing the attachment to and penetration of the susceptible erythrocytes by the organism. For this purpose, the techniques available for tracing antigens with labeled antibodies deserve attention.

SUMMARY

The initial and polyhedral body stages of *Anaplasma marginale* were liberated into the lysate of blood from acutely infected cattle by mechanical disintegration and autoantibody action upon the erythrocytes. The lysates from the blood of infected animals were filtered through a filter with an average pore diameter of 0.65 μ into freshly collected whole blood from animals free of anaplasmosis. Subsequent examination of this blood with an electron microscope revealed initial bodies attached to erythrocytic membranes and initial and polyhedral bodies that were not attached to cells. Anaplasma-free blood, when admixed with filtered lysate containing initial Anaplasma bodies and injected into animals from which the blood was originally obtained, resulted in anaplasmosis infection. The infection was demonstrated by clinical signs, development of complement-fixation titers, and appearance of marginal bodies within the erythrocytes.

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SUMMARIO IN INTERLINGUA

Studios de Anaplasmosis. I. Filtration del Agente Causative

Anaplasma marginale, in le stadios corporee initial e polyhedral, esseva liberate a in le lysato de specimens de sanguine ab acutemente inficite bestial per medio de disintegration mechanic e del action de autoanticorpore super le erythrocytos. Le lysatos ab le sanguine de inficite animales esseva filtrate per un filtro con poros de un diametro medie de 0,65 μ a in frescamente colligite sanguine total ab animales libere de anaplasmosis. Le subsequente examine de iste sanguine per medio de un microscopio electronic revelava corpores initial attachate a membranas erythrocytic e corpores initial e polyhedral que non esseva attachate a cellulas. Sanguine libere de anaplasmosis, quando admiscite al lysato filtrate que contineva corpores initial de *Anaplasma* e re-injicite in le animales ab le quales illo habeva essite obtenite, resultava in un infection anaplasmotic. Iste infection esseva demonstrate per signos clinic, le disveloppamento de titros de fixation de complemento, e le apparition de corpores marginal intra le erythrocytos.

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A Rapid Method for Determining the Viability of *Fasciola hepatica* Metacercariae

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In screening various physical or chemical methods for their ability to destroy the infective stage of *Fasciola hepatica*, the viability of metacercariae is determined by animal inoculation. However, even the relatively rapid mouse test, developed by Taylor and Parfitt,[7] requires a waiting period of three weeks. Moreover, since 1 mouse is required for each metacercaria and viability is determined at necropsy, testing numerous metacercariae is time-consuming.

Artificial digestion, which imitates some natural conditions in the host, stimulates the infective stage of the helminth. Furthermore, its effect is easily observable from its very beginning.

The use of artificial digestion for the determination of viability has already been studied in helminthology. Silverman [5] developed such a technique for the ova of several species of Taenia; Meyers,[2] for ova of *Echinococcus granulosus*; Mikačić,[3] for the scolices of *E. granulosus*; and Kozhemyakin and Shlyakhtenko,[1] for *Cysticercus cellulosae*.

In the present study, we investigated the action of artificial digestion upon *Fasciola hepatica* metacercariae in an attempt to develop a rapid, simple, and economical method for determining their viability.

Materials and Methods

After preliminary trials with various artificial digestive juices, we adopted a pepsin solution containing 0.5 Gm. of pepsin (activity, 1:3,000 U.S.P.), 0.8 Gm. of NaCl, and 100 ml. of n/20 HCl, and a trypsin solution containing 0.4 Gm. of trypsin (activity, 1:250 U.S.P.), 0.8 Gm. of NaCl, 1.0 Gm. of $NaHCO_3$, and 100 ml. of distilled water. The action of trypsin solution alone and trypsin solution containing 10 per cent ox bile, 20 per cent ox bile, or whole ox bile was studied. The action of these mediums was also studied after pretreatment with pepsin solution, the treatment time with the latter being variable. The action of the pepsin solution alone and of pepsin solution followed by the addition of whole ox bile or physiologic saline solution was also considered.

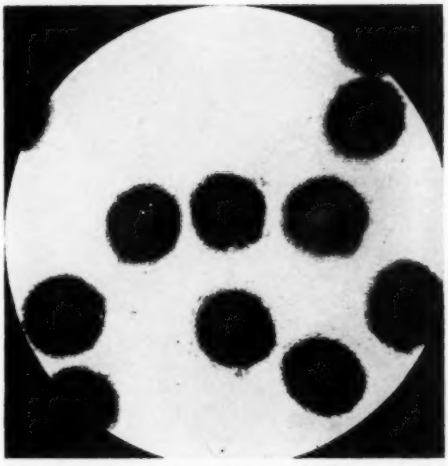

Fig. 1—Intact metacercariae attached to cellophane. Approximately x 60.

Fasciola hepatica eggs were obtained by incubating live worms in 0.9 per cent physiologic saline solution at 38 C. for a few hours. Eggs were suspended in tap water and kept in darkness at 25 C. for 12 days to develop miracidia. Hatching was induced by exposure to light. Half-grown snails *(Galba truncatula)*, raised in the laboratory on mud slopes according to the method of Taylor and Mozley [6] or on sand moistened with pond water and enriched with dead leaves of iris, were infected with 5 to 10 miracidia each. The emergence of

Fig. 2—Activated metacercariae moving to rupture the cysts. Approximately x 120.

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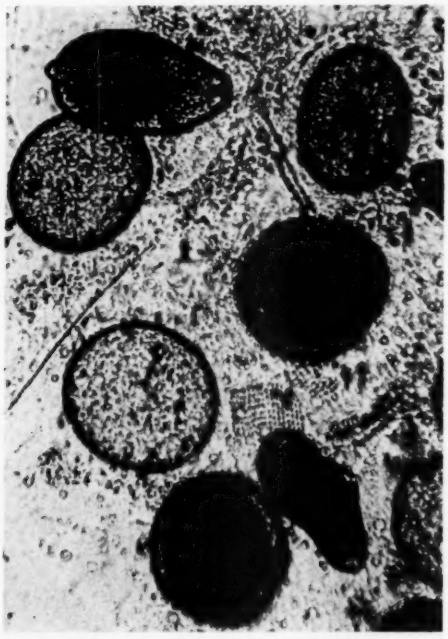

Fig. 3—Excysted metacercariae moving among non-excysted ones and among empty cysts. The outer layer of the cysts has been completely digested. Approximately x 135.

cercariae was induced eight to ten weeks later by placing the snails into tubes containing water and lined with cellophane, as recommended by Urquart.[5]

The testing was performed in watch glasses, preferably in 5-ml. depressions ground in glass blocks, into which a digestive medium was poured and warmed to approximately 38 C. A small piece of cellophane, with 4- to 7-day-old metacercariae attached (fig. 1), was then immersed in the medium. The material was covered and placed in an incubator. Microscopic examination was performed in a Foot's chamber, insuring a practically constant temperature of 38 C. Observations were started ten minutes after immersion and continued for 48 hours. If the metacercariae were treated successively with two mediums, the second one was added in equal amount.

In order to study the survival of excysted metacercariae in a nonproteolytic medium, the artificial digestive juice which caused excystation was carefully pipetted off and simultaneously replaced by warmed Tyrode's solution containing 200 units of penicillin and 0.2 mg. of streptomycin per milliliter of solution.

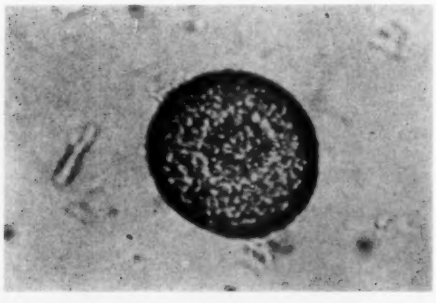

Fig. 4—An empty cyst (inner layer only) showing a circular opening through which the metacercariae had emerged. Approximately x 150.

RESULTS AND DISCUSSION

Without pretreatment with pepsin solution, the trypsin solution, either alone or containing ox bile, did not cause any change in appearance or behavior of the metacercariae.

Under the action of the pepsin solution, the outer layer of the cyst underwent partial digestion, rendering the cyst transparent so that the rare and weak movements inside the cyst were observable. Although in some cases this process started 45 minutes after immersion, in the majority of metacercariae it was observed only in the second and third hour of pepsin treatment. However, complete excystation never occurred in the pepsin solution, even if the treatment lasted as long as 48 hours.

If the trypsin solution was added to the pepsin solution after the latter had been allowed to act for two to three hours, the metacercariae became active (fig. 2) and eventually excysted (fig. 3). The outer layer of the cyst usually disintegrated entirely. The highest rate of excystation (about 80 %) was observed in a trypsin medium containing 20 per cent ox bile. It was somewhat lower (about 64%) in a trypsin medium containing 10 per cent ox bile, and was much lower (under 1%) in the trypsin solution without bile. If the pepsin pretreatment was followed by the addition of whole ox bile or physiologic saline solution alone, excystation did not occur.

The highest percentage of metacercariae excysted in the second and third hour of the treatment with trypsin solution and 20 per cent ox bile, although some of them excysted after 15 minutes. Metacercariae excysted through a circular opening approximately 65 μ in diameter (fig. 4).

After excystation, the metacercariae moved actively for one to two hours; if the treatment was continued, they became ex-

hausted, died, and disintegrated. Their empty cysts, however, resisted the proteolytic activity of the experimental mediums for at least 48 hours, which is in accordance with Schumacher's [4] *in vivo* observations.

If the digestive medium was replaced by Tyrode's solution after excystation had been induced, the excysted metacercariae survived readily up to 42 hours.

CONCLUSION

Active excystation of viable metacercariae was induced by use of suitable artificial digestive medium. Excystation only occurred in trypsin mediums, but pretreatment with pepsin solution was indispensable. The addition of ox bile to the trypsin solution enhanced the rate of excystation considerably.

SUMMARY

A rapid, simple, and economical method for determining the viability of *Fasciola hepatica* metacercariae was developed by studying the effects of various artificial digestion mediums. Active excystation of viable metacercariae was induced by treating them first with pepsin solution (0.5 Gm. of pepsin, activity 1:3,000 U.S.P.; 0.8 Gm. of NaCl; and 100 ml. of n/20 HCl) for two to three hours and then with trypsin solution (0.4 Gm. of trypsin, activity 1:250 U.S.P.; 0.8 Gm. of NaCl; 1.0 Gm. of $NaHCO_3$; and 100 ml. of distilled water) supplemented with 20 per cent ox bile for an additional two to three hours.

By replacing the digestive medium with Tyrode's solution, the survival of excysted metacercariae was readily prolonged to 42 hours.

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SUMMARIO IN INTERLINGUA

Un Rapide Methodo pro Determinar le Viabilitate de Metacercarias de Fasciola hepatica

Un rapide, simple, e incostose methodo pro determinar le viabilitate de metacercarias de *Fasciola hepatica* esseva disveloppate per studiar le effectos de varie medios artificial de digestion. Excystation active de viabile metacercarias es inducite per tractar los primo, durante duo a tres horas, con un solution de pepsina (0,5 g de pepsina, activitate 1:3.000, U.S.P.; 0,8 g de NaCl; e 100 ml de N/20 HCl) e postea, de novo durante duo a tres horas, con un solution de trypsina (0,4 g de trypsina, activitate 1:250, U.S.P.; 0,8 g de NaCl; 1,0 g de $NaHCO_3$; e 100 ml de aqua distillate) supplementate con 20 pro cento de bile de bove.

Per reimplaciar le medio de digestion per solution de Tyrode, le superviventia del excystate metacercarias esseva facilemente prolongate a 42 horas.

Studies of the Effects of Chlortetracycline, Vitamin E, and Vitamin K on the Nutrition of the Rat. II. Reproduction

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CHLORTETRACYCLINE and vitamin K have been found effective in partially relieving a dietary deficiency of vitamin E as required for the growth of rats; it has also been shown that testicular degeneration did not occur when rats were fed a vitamin E-deficient ration supplemented with chlortetracycline.[6] Female rats of the previous study [6] were maintained on diets with variable quantities of vitamin E, vitamin K, and chlortetracycline. The effects of these dietary variables on body weight, reproductive behavior, life span, and liver vitamin A content are the subject of this report.

PROCEDURE

The experimental design, description of animals, and ration composition were previously reported.[6] Approximately 60 per cent of the calories supplied by the diet were derived from lard. A factorial design was used involving three variables: vitamin E (E), vitamin K (K), and chlortetracycline (A). The vitamins and chlortetracycline were fed at levels of 0.02 per cent and 0.03 per cent of the ration in replications 1 and 2, respectively. Replication 1 had 4 and replication 2 had 5 female rats per treatment. The rats of replication 1 were fed the experimental rations from 3 to 55 weeks of age, and rats in replication 2 from 3 to 104 weeks of age. The males were fed commercial pellets when not being used for mating purposes. Mating was started at the end of experimental week 11 in both replications. A male was caged with each female for one week and was replaced by a second male for the second week and a third male for the third week. Each three-week mating period signifies one mating (tables 1, 2).

The female rats were observed daily for birth of young. They were weighed at weekly intervals and were given fresh feed three times each week. All rats had free access to feed and water. The surviving young were weaned at 21 days of age and fed the respective test rations for the next seven days. The effects of vitamin K and E and of chlortetracycline on gestation weights, number of young born and weaned, weaning weights, weights at 28 days of age, weight changes of the females during lactation, and the weights of the females at experimental week 39 and at 1 year were determined. The body weights of each rat for experimental weeks 50 through 55 were used to calculate the average body weight for one experimental year.

RESULTS AND DISCUSSION

Replication 1.—The data on the reproduction of the females are presented (table 1). None of the females fed the basal ration (treatment O) gave birth to young. Rats fed this ration showed signs of vitamin E deficiency. They became thin and emaciated and had difficulty in walking due to lack of coordination of the hindlegs. These rats did not walk on the toes and feet of their hindlimbs, but on the lower part of the leg, foot, and toes; function of the front feet appeared normal.

No young were born following nine of 11 matings in treatment K, eight of the ten matings in treatment A, and eight of the 11 matings in treatment AK. Additional matings of rats in the K, A, or AK treatments resulted in the birth of seven litters (table 1). However, the young did not

TABLE 1—Effect of Vitamin E, Vitamin K, and Chlortetracycline on Reproduction of Female Rats (First and Second Litters; Replication 1)

	Treatment							
	O*	K	A	AK	E	EK	EA	EAK
No. times mated	11	11	10	11	8	8	9	7
No. litters	0	2	2	3	8	8	7	7
No. young	....	5	12	21	66	68	81	66
No. young weaned	....	0	0	0	12	7	24	37
Av. weaning weight (Gm.)	....	....	....	....	32	34	36	44
Av. 28-day weight (Gm.)	....	....	....	....	46	41	56	59
Av. weight change of female during lactation (Gm.)	....	....	....	....	−8	+5	−10	−23

* O = Deficient in vitamins E(E) and K(K) and lacking in chlortetracycline (A).

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survive to weaning age. This indicated that 0.02 per cent vitamin K or chlortetracycline partially relieved the dietary deficiency of vitamin E to the extent that the female was capable of carrying her young to term and giving birth to them. The number of litters and young born to those females receiving the combination of A and K was greater than the number born to the females receiving either A or K alone. A similar effect of vitamin K has been reported by Jensen *et al.*,[3] who found that the addition of vitamin K to a vitamin E-deficient ration increased the hatchability of fertile turkey eggs by 8.5 per cent.

The total number of young born in the E, EAK, and EK treatments was about the same and all matings resulted in the birth of young. Rats fed the EA ration delivered the largest number of offspring per litter and seven of nine matings resulted in the birth of young.

Replication 2.—Data on the reproduction of the females are presented (table 2). None of the females fed the basal ration (treatment O) gave birth to young. When 0.03 per cent vitamin K was added to the basal ration, 14 matings resulted in the birth of three litters. There were 12 mating opportunities in treatment A in which four litters were born. Rats fed the AK ration gave birth to first-litter young which survived to weaning age but the young born in their second litters did not reach this age. Rats in one litter of 6 young in treatment AK developed rough coats and appeared unable to coordinate their hindlegs about two days after weaning. Three young died during the second day, 1 the third day, and 2 the fourth day following weaning. These young showed signs of a vitamin E deficiency as described by Poling *et al.*[5] This indicates vitamin K and chlortetracycline delayed the effects of the vitamin E deficiency.

The addition of vitamin E to the basal ration in treatments E, EK, and EAK resulted in the birth of young. In treatment EA, 13 matings resulted in the birth of ten litters.

The reproductivity of the females in replication 2 was much better than that of females in replication 1. In replication 1, *dl-alpha* tocopheryl acetate was included in the ration at a level of 0.02 per cent. Since 1 mg. of *dl-alpha* tocopheryl acetate is equivalent to 1.0 I.U. of vitamin E,[4] the rats received 0.2 I.U./Gm. of ration. In replication 2, *d-alpha* tocopheryl acetate was used in the ration at a level of 0.03 per cent. Since 1 mg. of *d-alpha* tocopheryl acetate is equivalent to 1.36 I.U. of vitamin E, the rats received 0.4 I.U./Gm. of food. Although this ration had a high caloric density, a 5-Gm. daily feed intake per rat in replication 2 would have supplied 2 I.U. of vitamin E.

TABLE 2—Effect of Vitamin E, Vitamin K, and Chlortetracycline on Reproduction of Female Rats (First and Second Litters; Replication 2)

	Treatment							
	O*	K	A	AK	E	EK	EA	EAK
No. times mated	15	14	12	12	10	10	13	8
No. litters	0	3	4	6	10	10	10	8
No. young	0	24	35	34	93	101	97	85
No. young weaned	0	0	5	10	42	38	39	47
Av. weaning weight (Gm.)	....	....	40	36	31	38	30	35
Av. 28-day weight (Gm.)	....	....	....	....	47	58	47	57
Av. weight change of female during lactation (Gm.)	....	....	+6	+12	−15	−17	−25	−57

* O = Deficient in vitamins E(E) and K(K) and lacking in chlortetracycline (A).

Luckey *et al.*[4] were unable to produce prolongation of the blood-clotting time in germ-free rats on a vitamin K-deficient diet for 400 days. However, Gustafsson [2] has shown that a bleeding tendency and prolonged prothrombin times occurred in germ-free rats within five to nine days when they were fed a vitamin K-deficient diet. In the experiment reported here, there was no bleeding tendency in any of the rats. They probably acquired sufficient vitamin K coprophagally to prevent it.

Chlortetracycline (0.02%) relieved the dietary deficiency of vitamin E to the ex-

TABLE 3—Effect of Vitamin E, Vitamin K, and Chlortetracycline on the Average Body Weights (Gm.) of the Female Rats at the Fourth Week After the Initial Mating Opportunity

Treatment	Replication	
	1	2
O*	225	225
K	272	278
A	268	300
AK	299	297
E	342	306
EK	337	321
EA	314	357
EAK	329	375

* O = Deficient in vitamins E(E) and K(K) and lacking in chlortetracycline (A).

TABLE 4—Effect of Vitamin E, Vitamin K, and Chlortetracycline on the Average Body Weights (Gm.) of the Female Rats Fed the Rations for One Year

Treatment	Replication	
	1	2
O	205	238
K	303	322
E	352	310
EK	346	360
A	254	294
AK	365	305
EA	343	358
EAK	339	386

tent that five matings yielded birth of young. When 0.03 per cent chlortetracycline was added to the vitamin E-deficient ration, the young of one litter survived to weaning age, indicating that chlortetracycline had some ability to overcome the effects of the vitamin E-deficient ration. Chlortetracycline or vitamin K overcame the effects of vitamin E deficiency to the extent that 16 per cent (K), 23 per cent (A), and 35 per cent (AK) of the matings resulted in the birth of live young. However, the reproduction data of the rats in treatment E, replications 1 and 2, indicate chlortetracycline is not necessary for reproduction. These data also indicate it is not necessary to include vitamin K in the ration, as fed in this experiment, for reproduction.

Table 3 contains the peak of the body weights during gestation following the first and second matings combined for each treatment for both replications. The peak of the average body weights for all matings occurred four weeks following the first day of breeding. The addition of vitamin K to the basal ration had no significant effect on body weights during gestation. The addition of chlortetracycline or vitamin E to the basal ration significantly ($P = 0.01$) increased the body weights of the pregnant rats.

There was no significant difference, due to magnitude of treatment, on body weight gains through week 39 in replication 1 compared with replication 2, justifying their combination for statistically testing treatment effects. The average weight gain of rats fed vitamin E was significantly ($P < 0.001$) greater than that of rats not receiving vitamin E, these averages being 252 and 217 Gm., respectively. Vitamin K and chlortetracycline each had an effect, as indicated by their interaction being significant ($P = 0.01$). They were about equally beneficial, but their combination was not appreciably superior to either one alone. The average gains for those receiving treatment O, vitamin K alone, chlortetracycline alone, and vitamin K with chlortetracycline were 205, 245, 239, and 248 Gm., respectively. A growth-stimulating effect of an antibiotic has also been reported by Luckey *et al.*,[4] who found it increased the growth rate of germ-free turkey poults.

The average body weights of the rats fed the various rations for one year were recorded (table 4). The addition of vitamin E or K to the basal ration had a significant effect in both replications. In replication 1, the addition of chlortetracycline had a significant ($P = 0.001$) adverse effect but in replication 2 it had no effect, the disagreement being significant ($P = 0.01$). There was a significant ($P = 0.01$) interaction between vitamins K and E; *i.e.*, K and E together resulted in far better weight gains than would be expected from their separate effects.

There were no significant treatment effects on life span in replication 2. Fourteen rats were killed after being on the experiment for two years. Twenty-six rats died during the two-year period and their average life span was 73 weeks.

SUMMARY

Vitamin K and chlortetracycline were effective in overcoming vitamin E deficiency to the extent of permitting female rats to bear young. Chlortetracycline was effective in overcoming vitamin E deficiency to a greater extent than was vitamin K in that it enabled the females to bear young and raise them to weaning age. The average weight gain of rats fed the basal ration plus vitamin E was significantly greater than that of rats not receiving vitamin E. Vitamin K and chlortetracycline increased the body weight gains significantly and about equally.

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SUMMARIO IN INTERLINGUA

Studios Relative al Effectos Producite per Chlortetracyclina, Vitamina E, e Vitamina K in le Nutrition del Ratto
II. Reproduction

Vitamina K e chlortetracyclina se monstrava capace a superar carentia de vitamina E usque a un grado permittente parturir prole a rattos feminin. Chlortetracyclina se monstrava capace a superar carentia de vitamina E usque a grados plus alte que vitamina K. Illo permitteva a rattos feminin parturir prole e elevar lo usque al etate de dislactation. Le augmento medie del peso de rattos recipiente le dieta basal plus vitamina E esseva significativemente plus grande que illo de rattos non recipiente vitamina E. Vitamina K e chlortetracyclina accelerava le augmento del peso corporee significativemente e plus o minus equalmente.

The Use of Tricalcium Phosphate for the Adsorption and Concentration of Erysipelas Bacterin

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THE USE of calcium salts to concentrate and purify bacterial antigens was first reported by Roux and Yersin[7] in 1889. Since then, calcium phosphate has been employed by various investigators[1-6] to purify and concentrate antigen and antibody preparations.

Traub,[8] in 1947, used aluminum hydroxide (Al [OH] $_3$) to adsorb and concentrate *Erysipelothrix rhusiopathiae* culture for the purpose of making a bacterin. This bacterin, since known as Traub's adsorbate bacterin, has been used extensively and with good results in this country and Europe.

The work reported here was initiated because of our observations that a specially prepared colloidal suspension of tricalcium phosphate ($Ca_3[PO_4]_2$)* has the ability to adsorb and concentrate certain solutes and colloidal material, such as erysipelas culture, to a greater degree than aluminum hydroxide gel.

MATERIALS AND METHODS

The tricalcium phosphate (control No. 3340, Ca = 40.87%, P = 17.88%) was manufactured for the property of maximum adsorption. A 2 per cent suspension (ash weight; 800 C., 2 hours), pH 6.7 was prepared for this experiment.

The aluminum hydroxide was purchased on the market as a compressed gel (11.1%, expressed as Al_2O_3) manufactured for its adsorptive quality and commonly used in this country in the manufacture of erysipelas bacterin. A 2 per cent suspension, pH 7.5, was prepared for this experiment.

Experiment 1, Quantitative Adsorption of Congo Red by Tricalcium Phosphate and Aluminum Hydroxide.—The adsorbents were tricalcium phosphate and aluminum hydroxide; the adsorbate was Congo red,* with total dye content 92 per cent (certification no. N.Q. 16, Biological Stain Commission).

A series of 14 tubes were set up in duplicate for this study. Twenty-five milliliters of a Congo red solution, with an arbitrary concentration of 770 μg. per milliliter (equivalent to 19.25 mg. per tube), was pipetted into each tube. Graduated amounts of 2 per cent aluminum hydroxide, ranging from 0.5 to 8.0 ml. (equivalent to 10 to 160 mg. ash weight per tube), were pipetted into tubes 1 to 7. Graduated amounts of 2 per cent tricalcium phosphate suspension ranging from 0.5 to 8.0 ml. (equivalent to 10 to 160 mg. ash weight per tube), were pipetted into tubes 8 to 14. Distilled water was added to each tube to make the volume 33 ml. per tube. A distilled water system was used for this test rather than a buffered solution since the electrolyte effect of the buffer salts reduced the adsorptive capacity of both gels.

The tubes were shaken vigorously by hand for five minutes, as this was sufficient for maximum gel dispersion and adsorption. Samples taken from the tubes were centrifuged at 2,500 r.p.m. to remove all gel particles plus the adsorbed Congo red. The remaining Congo red in the supernatant fluid was assayed by determination of the optical density with the Beckman DU Model B spectrophotometer at a wave length of 497 Å.

The maximum absorption of Congo red may vary at different pH values and thus affect measurements based on optical density. The minor pH differences among solutions assayed in this experiment were shown to be incidental in this regard.

Experiment 2, Adsorption and Adjuvant Studies with Erysipelas Culture.—Four separate immunogenic strains of *Ery. rhusiopathiae** were grown individually in a medium of horse meat and horse liver infusion with 2.0 per cent peptone, 1.1 per cent phosphate salts, 5.0 per cent horse serum, and 1.0 per cent glucose at a final pH of 8.0. At the end of 36 hours of growth at 37 C., the cultures were pooled.

The pool was divided into three aliquots and placed in 1,500-ml. test tubes (fig. 1). Two per cent tricalcium phosphate was added to the first aliquot in an amount equal to 25 per cent of the culture and the tube was shaken vigorously. This

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* Neojel, produced by Diamond Laboratories, Des Moines, Iowa.

* Manufactured by the National Aniline Division of Allied Chemical and Dye Corp., New York, N.Y.

* Two of the strains were obtained from Dr. K. Von Bakos, State Veterinary Institute, Stockholm, Sweden, and two were obtained from Dr. R. D. Shuman, Agricultural Research Service, USDA, Washington, D.C.

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suspension was allowed to settle for 96 hours at room temperature and the clear supernatant fluid was removed. The precipitate (antigen-tricalcium phosphate concentrate) represented 2/15 of the original volume of culture. This is the material normally contained in a 2-cc. dose of erysipelas bacterin.

Two per cent aluminum hydroxide was added to the second aliquot in an amount equal to 25 per cent of the culture and the tube was shaken vigorously. This suspension was also allowed to settle for 96 hours at room temperature and the clear supernatant fluid was removed. The precipitate (antigen-aluminum hydroxide concentrate) represented 1/3 of the original volume of culture. This is the material normally contained in a 5-cc. dose of erysipelas bacterin.

In preparation for the mouse potency test, the two culture concentrates were diluted with distilled water to their original volume so that they would contain presumably the same concentration per milliliter of antigen as the original cultures. The assumption was made that the gels had completely adsorbed the antigen.

The mouse potency test (table 2), based on a test set forth by the U.S. Department of Agriculture,* consists of immunizing 16 to 18 mice at each three serial (threefold) dilutions with the bacterin to be tested and with the USDA standard bacterin. The mice are given 0.5 ml. of the bacterin dilution subcutaneously and, two weeks later, the immunity of 16 mice at each dilution is challenged with approximately 1,000 m.l.d. of *Ery. rhusiopathiae*, strain EW_2. The bacterins may be compared on the basis of total number of mice protected.

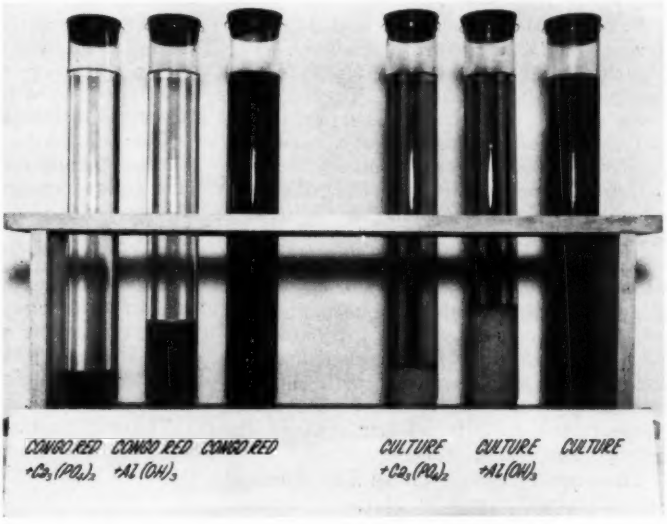

Fig. 1—The adsorption and concentration of Congo red and erysipelas whole culture by $Ca_3(PO_4)_2$ and $Al(OH)_3$ at 96 hours.

Previous determinations indicated that both gels concentrate practically all antigen. Therefore, it is important to notice that the 2-cc. dose of antigen-tricalcium phosphate concentrate and the 5-cc. dose of antigen-aluminum hydroxide concentrate have equal amounts of antigen. The third aliquot was held unconcentrated. At the same time, and in a similar manner, the gels were added to Congo red (770 μg./ml.) for comparison purposes (fig. 1).

The supernatant fluid was removed from the two tubes containing adsorbed erysipelas culture and the concentrates were saved for potency tests on mice. The supernatant fluid from the tricalcium phosphate-adsorbed culture was readsorbed with tricalcium phosphate, and this supernatant concentrate was saved for the mouse potency test to determine if the protective antigen was entirely removed by the first adsorption.

RESULTS AND DISCUSSION

Experiment 1, Quantitative Adsorption of Congo Red by Tricalcium Phosphate and Aluminum Hydroxide.—The amount of Congo red adsorbed at each level of gel was calculated (table 1). It may be seen that 120 mg. (ash weight) of tricalcium phosphate gel, equal to 0.384 millimoles, adsorbed approximately the same amount of Congo red as aluminum hydroxide with an ash weight of 120 mg., equal to 1.536 millimoles.

* United States Department of Agriculture, ARS circular letter ZV, November 18, 1953.

TABLE 1—Comparative Adsorption of Congo Red by Tricalcium Phosphate and Aluminum Hydroxide

$Ca_3(PO_4)_2$			$Al(OH)_3$		
Ash weight adsorbent (mg.)	Millimoles adsorbent	Congo red adsorbed (mg.)	Ash weight adsorbent (mg.)	Millimoles adsorbent	Congo red adsorbed (mg.)
10	0.032	1.43	10	0.128	3.87
20	0.064	4.06	20	0.256	7.13
40	0.128	9.57	40	0.512	12.81
60	0.192	14.04	60	0.768	17.68
80	0.256	18.35	80	1.024	19.13
120	0.384	19.23	120	1.536	19.25
160	0.512	19.25	160	2.051	19.25

The adsorption of Congo red by aluminum hydroxide is superior to the tricalcium phosphate on an ash weight basis; however, on a molar basis, the tricalcium phosphate is superior.

Experiment 2, Adsorption and Adjuvant Studies with Erysipelas Culture.—The adsorbing and concentrating ability of the two gels is shown (fig. 1). They concentrate soluble substances, such as Congo red, as well as particulate material such as the *Ery. rhusiopathiae* organisms. As seen in evidence presented later, the tricalcium phosphate gel also concentrates the soluble erysipelas antigens.

By column measurements made at the end of the 96-hour settling period, it was shown that the tricalcium phosphate-antigen complex concentrated to approximately 13 per cent of the original suspended culture volume, as contrasted with the aluminum hydroxide-antigen complex which concentrated to only 33 per cent of the original suspended culture volume.

These concentration values were obtained by natural settling in 96 hours. Additional settling after longer periods occurred only to a minor degree. The concentration of each of the two antigen-gel complexes represents the respective limiting degree of viscosity practical for injection purposes. The tricalcium phosphate has the advantage of combining an equal amount of antigen in a smaller volume. The same results were obtained with Congo red adsorption as with the antigen suspensions.

TABLE 2—Adjuvant Effect of Aluminum Hydroxide and Tricalcium Phosphate on Erysipelas Bacterin in Mice

Immunizing bacterin*	Immunizing antigen dilutions			
	1:16	1:50	1:150	Totals
$Al(OH)_3$ adsorbed	13/16**	13/16	10/16	36/48
$Ca_3(PO_4)_2$ adsorbed	16/16	13/16	9/16	38/48
Unconcentrated culture	7/16	5/16	3/16	15/48
Supernatant after adsorption with $Ca_3(PO_4)_2$ and readsorbed with $Ca_3(PO_4)_2$	3/16	1/16	0/16	4/48

* Prior to diluting the antigens, distilled water was added to the two adsorbed cultures proportional to their original concentration so that they presumably contained the same quantity of antigen as the unconcentrated culture.

** Sixteen mice were immunized subcutaneously with each product at each antigen dilution and challenged two weeks later. Survivors/total are recorded at seven days postchallenge.

The tricalcium phosphate-adsorbed erysipelas culture and the aluminum hydroxide-adsorbed erysipelas culture gave about the same degree of protection to mice (table 2). Both gels also had an adjuvant effect. Of 48 mice vaccinated with the untreated culture, only 15 were protected against challenge; of 48 mice vaccinated with tricalcium phosphate-adsorbed culture, 38 were protected; and of 48 vaccinated with aluminum hydroxide-adsorbed culture, 36 were protected.

The supernatant fluid from the tricalcium phosphate-adsorbed erysipelas culture has very little antigen remaining (table 2). This supernatant fluid, readsorbed with the tricalcium phosphate in the same manner as the original culture and tested on mice, protected only 4 of 48 mice against challenge.

SUMMARY AND CONCLUSIONS

As an adjuvant and concentrating agent, the merits of tricalcium phosphate were compared with those of aluminum hydroxide. Tricalcium phosphate gel was shown to concentrate erysipelas antigen more effectively than aluminum hydroxide, within the limits of usable viscosity. A desirable and similar adjuvant effect was demonstrated for both gels. In combination with erysipelas antigen, they increased the level of immunity in mice to *Erysipelothrix rhusiopathiae* challenge beyond that obtained with the antigen alone.

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SUMMARIO IN INTERLINGUA

Le Uso de Phosphato Tricalcic in le Adsorption e Concentration de Bacterina de Erysipelas

Como agente adjuvante e concentratori, le meritos de phosphato tricalcic esseva comparate con illos de hydroxydo de aluminium. Esseva trovate que gel de phosphato tricalcic effectua un plus efficace concentration de antigeno de erysipelas que hydroxydo de aluminium, intra le limites del viscositate usabile. Un desirabile e similemente adjuvante effecto esseva demonstrate in le casos de ambe gels. In combination con antigeno de erysipelas illos augmentava le nivello del immunitate de muses contra le provocation con *Erysipelothrix rhusiopathiae* in ultra del nivello obtenite per le antigeno sol.

Studies on the Pharmacology of N-(ethylmercuri)-*p*-toluenesulfonanilide

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VARIOUS ORGANOMERCURIAL compounds are commonly used as seed disinfectants for control of fungus parasites of grain. The pathologic effects of some of these compounds have been reported for cattle,[6,12,14,35] poultry,[7,13,15] laboratory animals,[37] swine,[21,38] and man.[22,44] However, there have been no reports of the progressive cumulative effects of any of these compounds upon various body systems, and only a few limited accounts of their disposition in the body.

MATERIALS AND METHODS

N-(ethylmercuri)-*p*-toluenesulfonanilide (EMTS) was administered in a commercial preparation (Ceresan M*) which contains 7.7 per cent of the compound. The compound was dispensed into gelatin capsules at the prescribed dosages and administered orally.

Calves, approximately 6 months old, were used as experimental animals. Animal 1, a Jersey heifer, was given EMTS daily at a calculated rate of 0.0112 Gm. per kilogram of body weight; animal 2, a Jersey steer, was given 0.056 Gm./kg. daily; and animal 3, a Holstein-Friesian heifer, was given 0.112 Gm./kg. daily. Animal 4, a Holstein-Friesian steer, was given a single 0.140 Gm./kg. dose, and animal 5, a Holstein-Friesian heifer, was given a single 0.224 Gm./kg. dose.

Electrocardiograms were run on a Sanborn Visocardiette, using standard leads with the subject (animal 1) in a standing position, except for the final graph which was made with the animal lying on its right side.

Electropherograms** of serum proteins were made in a Durrum-type Spinco hanging strip cell. The fractions were separated and stained by the method of Block *et al.*[4] After staining, the strips were scanned on a Spinco Analytrol photoelectric cell, and the results were tabulated as percentages of the sum of all the areas.

To study the *in vitro* binding of EMTS by plasma albumin, 100 ml. of blood plasma from an untreated animal was incubated at 37 C. with 38.5 mg. of EMTS for 90 minutes. Excess EMTS was removed from the sample by centrifugation at 10,700 r.p.m. for 30 minutes in a Spinco Model K preparative centrifuge.

The total protein[34] and mercury[36] concentrations and the relative albumin value in the centrifuged plasma were determined.

Using 50 ml. of the centrifuged sample, the albumin fraction was separated by the method of Milch;[23] the mercury and albumin concentrations in the final aqueous dialysate were then established.

The relative purity of the albumin was determined electrophoretically after a portion of the aqueous dialysate was further dialyzed against veronal buffer (pH 8.6) to prevent diffusion during the electrophoretic procedure.

The actual albumin concentration in the plasma was calculated from the values obtained for the total protein and relative albumin concentrations. The albumin-bound EMTS was calculated from the difference between the mercury concentration in the centrifuged plasma and that in the aqueous dialysate.

Blood urea nitrogen was determined by the method of Van Slyke and Cullen[39]; packed-cell volume by the method of Wintrobe[43]; hemoglobin by the method of Sanford *et al.*[31]; blood chlorides by the method of Schales and Schales[32]; urine chlorides according to the Association of Agricultural Chemists[25]; and serum proteins by the method of Simmons.[34] Mercury was determined by the method of Oliver and Funnell,[26] and lead by "wet ashing" followed by a modified dithizon technique.[27]

RESULTS AND DISCUSSION

For 35 days there were no apparent changes in the appetite, excretions, or muscular coordination of animal 1. On day 36, there were signs of central nervous system involvement. The animal was incoordinate, the head was in almost continuous side-to-side motion, the eyelids twitched, and there were fine tremors of the muscles of the abdomen, limbs, and neck. Stimulation provoked tetanus-like spasms throughout the body. It was unable to coordinate its jaws sufficiently to eat or drink adequately and it salivated freely. On day 37, the animal was unable to rise but had some control over head movements; it made unsuccessful attempts to eat, but actively refused water. There were almost continuous paddling movements of the forelegs, and excitation produced tonic extensor spasms

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* The Ceresan M used in this investigation was supplied by E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.

** Paper electrophoretic fractionation patterns.

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and opisthotonos. The animal was killed on day 38 in an apparently terminal condition.

The pattern in animal 2 was similar, but the course of intoxication was shorter and the central nervous system was less acutely stimulated. The animal was essentially normal for 16 days after the start of drug administration. During the following week, its appetite became increasingly depressed and the bowel movements were scanty, but there were no abnormal cardiac or respiratory sounds. The first evidence of central nervous system derangement, head pressing, began on day 23. On the following day, fine muscular tremors developed and the animal was slightly incoordinate. There was excessive salivation, the respirations became shallow, and the pulse was poor. Death occurred on the 25th day.

In animal 3, the course of action of EMTS was brief. In this animal, the only clinical evidence of central nervous system involvement was asthenia which developed by the third day. On the fifth day, the animal was anorexic and the bowel movements became scanty. Rumen stasis was detected on the eighth day and the animal died on the ninth day.

Animal 4 did not appear to be affected by EMTS at any time during the experiment.

Animal 5 appeared normal for 60 hours after administration of the compound. Then there was a sudden onset of muscular weakness and tremors; salivation; rapid, shallow breathing; and teeth grinding. The animal died eight hours after development of clinical signs.

In these cattle, the predominant sign produced by EMTS was not consistent, but depended upon the rate of administration. With increased dosage, the character of the signs ranged from that of a primary central nervous system disturbance (animal 1) to a combined involvement of the central nervous and gastrointestinal systems (animal 2) to a primary gastrointestinal effect (animal 3). In the peracutely poisoned animal (5) there was no single dominant effect, the signs being those usually associated with general systemic collapse.

Absorption, Distribution, and Removal of EMTS.—A study was made of the absorption, distribution, accumulation, and removal of EMTS in cattle given single or multiple successive doses.

Absorption.—The latent period between the first administration and the appearance in the blood of detectable amounts of EMTS was inversely related to the size of the dose (fig. 1). At a dose rate of 0.0112 Gm./kg./day, animal 1 was given EMTS for 11 days before acquiring a blood level of 0.6 p.p.m. (expressed as mercury). The same level appeared in the blood of animal 5 (given 0.224 Gm./kg.) in less than six hours.

In each case, the log of the increasing level of the compound in the blood was linear with time, and the rate of increase was proportional to the increase in dose (fig. 2). The limit of the absorption rate was not reached by the ranges of EMTS administered in these experiments (fig. 2), although in both animals (4 and 5) given large single doses, the maximum concentration of EMTS in the blood was reached within 27 to 36 hours after administration (table 2).

The rate of absorption of EMTS in absolute amounts was found to increase at an increasing rate (fig. 1). Logarithmically there was a fixed rate of absorption for a

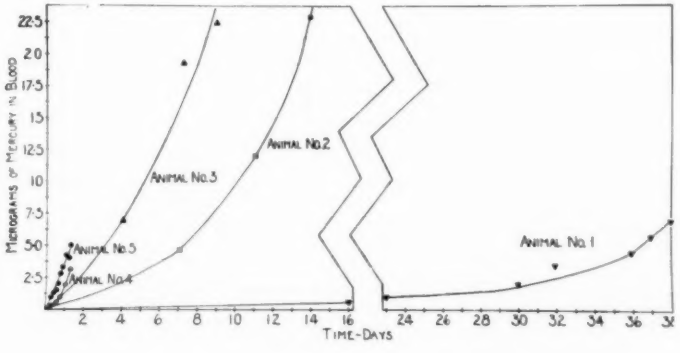

Fig. 1—The accumulation of mercury in the blood after the administration of single and multiple doses of N-(mercuri)-p-toluenesulfonanilide.

TABLE 1—Mercury Content of Tissues After Administration of N-(ethylmercuri)-p-toluenesulfonanilide

Organ	Animal No. 1	2	3	5
Kidneys				
Cortex	107.1	100.8	63.3	30.0
Medulla	14.0	129.6	60.5	27.8
Cerebrum	31.2	18.0	17.5	3.5
Cerebellum	51.2	.....	16.5	3.5
Medulla oblongata	15.4	.....	8.0	2.5
Spinal cord	19.5	.....	5.5	.....
Liver	50.0	108.0	58.1	46.5
Skeletal muscles	14.4	32.4	15.0	N.D.
Heart muscles	11.5	39.6	22.0	1.5
Spleen	16.5	54.0	36.5	11.6
Lungs	17.7	30.6	37.0	4.2
Duodenal wall	31.5	56.6	28.5	.....
Abomasal wall	8.5	16.2	18.0	.....
Perirenal fat	3.0	.....	7.0	.....
Mesenteric fat	3.7	.....	7.5	N.D.
Bile	3.0	.....	4.7	N.D.

Mercury content is given in micrograms per gram of wet weight. N.D. = not detectable.

given dose; this fixed rate increased linearly with increased dosages but the relationship differed, depending whether the drug was administered as a single dose or as multiple successive doses (fig. 3).

Transport.—The cells in 1 ml. of whole blood taken from animal 2 on day 14 contained 35.2 μg. of EMTS and the plasma contained 19 μg. Based upon the packed-cell volume (53.5 per cent at the time of sampling), 35 per cent of the drug in the blood was transported by the plasma and 65 per cent by the red blood cells.

Under the conditions described previously (*see* Materials and Methods), it was found that, *in vitro,* 1M of albumin bound with 2M of EMTS (0.00048M albumin: 0.00087M EMTS). If this value reflects blood saturation *in vivo,* the plasma albumin of animal 2 was not more than 12 per cent saturated with EMTS (0.00038M albumin: 0.000086M EMTS).

Distribution.—The compound was not uniformly distributed throughout the body (table 1). In each animal, the greatest concentration per gram of tissue was recovered from the kidneys (chiefly in the cortex of the more chronically poisoned animal 1, but essentially equally distributed between the cortex and medulla in the more acutely poisoned animals).

The extent of accumulation in nerve tissue was related to the duration of exposure rather than to the size of the dose. The nerve tissue of animal 1 contained higher levels of EMTS than comparative parts of animal 3, which in turn contained greater amounts than in the more acutely poisoned animal 5 (table 1). Although the greatest concentration in each animal was recovered from the kidneys, the trend indicated that, with extended exposure periods, the maximum levels would be expected to occur in nerve tissue. The mercurial was not equally distributed throughout various parts of this system. Concentrations in the cerebrum and cerebellum were similar in the more acutely poisoned animals (3 and 5) but, in the chronically poisoned animal (1), the cerebellum contained 1.7 times more mercury than the cerebrum. In all cases, the levels of the compound were greater in these two sites than any other parts of the central nervous system examined. Relating distribution to the decreasing duration of administration, the general order of concentration of EMTS in the central nervous system among individuals

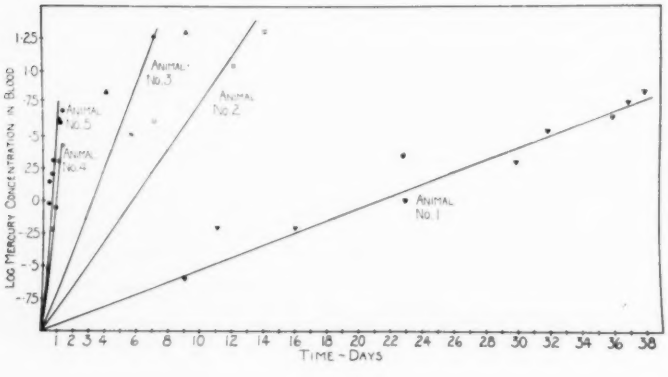

Fig. 2—The concentration of mercury in the blood, plotted semilogarithmically to show linearly the increase in accumulation with increased levels of administration of N-(mercuri)-p-toluenesulfonanilide.

was: cerebrum> cerebellum> medulla oblongata> spinal cord (table 1).

Within individuals, the distribution of the compound to other tissues was not regular, but in all cases was: liver> spleen> skeletal muscle> adipose tissue. In animal 5, given a single dose, the drug could not be detected in skeletal muscle or in the perirenal or mesenteric fat.

Removal.—The rate of removal of a nonsymptomatic dose of EMTS from the blood of animal 4 was dependent upon the concentration at any given time (table 2), taking the form of a "decay curve" (fig. 4) with the formula $Y = Y_o e^{-t/k}$.*

The linear relationship between time and the napierian value of concentration defined for the ideal process of drug removal did not apply in this experiment (fig. 5).

The rate of removal of an acutely toxic dose from the blood of animal 5 (table 2) was grossly distorted, taking the form of a parabola (fig. 4) with a k value of 38. This fourfold decrease in removal rate was interpreted to be the result of the toxic effect of the compound on the proximal tubules and glomeruli of the kidneys.

Cumulation.—From the formula $Y = Y_o e^{-t/k}$, the final level produced by n repetitions will equal $Y_n = \frac{Y_o}{1 - e^{-t/k}}$.**

The results of repeated administrations are presented (table 3). The predicted values given are based on the preceding formula and the actual levels after n repetition are based upon the amount recovered (in p.p.m.), an average blood volume of 5.8 per cent of the body weight [39] and a specific gravity of 1.052.[1]

In animal 1, the actual value exceeded the predicted value by 7 per cent. The discrepancy was considered to have resulted from the depressed excretion rate previously observed in animal 5 and discussed in the preceding section ("Removal"). In both animals, there were degenerative changes of the proximal tubules and glomerulonephritis.

In animals 2 and 3, the predicted values exceeded the actual by 30 per cent at a dose rate of 0.056 Gm./kg. and by 67 per cent at 0.112 Gm./kg. In this case, the decreasing ratio between the amount administered and the amount absorbed outweighed the accumulation tendency due to reduced exeretions. Multiple successive administrations of 0.0112 Gm./kg. produced an increasing drug concentration in the blood at a fixed rate of 1.13 μg./day; 0.056 Gm./kg. produced a fixed rate of 1.50 μg./day; and 0.112 Gm./kg. produced a fixed rate of 2.11 μg./day (fig. 3). Thus, a tenfold increase in the level of administration pro-

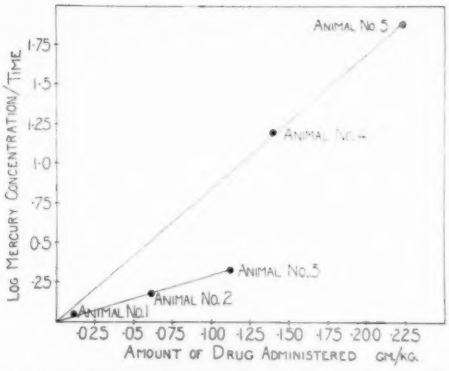

Fig. 3—The relationship of the fixed rates of absorption to the levels of administration of single and multiple doses of N-(mercuri)-p-toluenesulfonanilide.

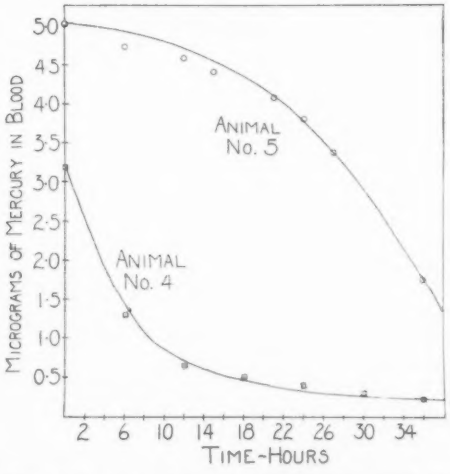

Fig. 4—A comparison of the rates of removal of mercury from the blood of animals with (5) and without (4) renal injury after single administrations of N-(mercuri)-p-toluenesulfonanilide.

* Y = concentration, t = time, Y_o = concentration at $t = o$, e = base of napierian logarithm, and k = constant for the system (time at which 63.2 per cent of the drug has disappeared); in this case, $k = 7$.

** Y_n = amount present after n repetitions, Y_o = amount of each administration, n = number of repetitions, t = interval between repetitions, and e = base of napierian logarithms.

TABLE 2—Accumulation and Removal of Mercury in Blood After Administration of N-(ethylmercuri)-p-toluenesulfonanilide

Time (hours)	Mercury (μg./ml.) Animal 4	Animal 5
0	N.D.	N.D.
3	....	0.6
6	0.3	0.95
9	....	1.4
12	0.6	1.6
15	....	2.0
18	0.9	2.3
21	....	3.4
24	2.0	4.3
27	....	4.1
30	3.25	5.1
36	1.4	4.7
42	0.65	4.6
45	....	4.4
48	0.5	....
51	....	4.1
54	0.4	3.8
57	....	3.4
60	0.3	....
66	0.25	1.8
72*	0.2	Animal dead

* Analyses were discontinued after 72 hours. N.D. = not detectable.

duced less than a twofold increase in absorption rate.

Mercury versus Lead.—The EMTS used in these experiments contained approximately 0.02 per cent lead. Since lead could have contributed to the signs, the tissues of animals 1, 2, 3, and 5 were assayed for this metal. None was recovered, even after 38 consecutive daily doses of the lead-containing compound. The failure of lead to appear in the tissues of cattle poisoned with lead arsenate also has been observed.[28]

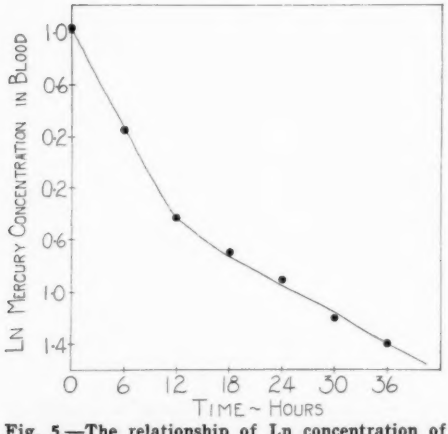

Fig. 5.—The relationship of Ln concentration of mercury in blood to time after a single administration of N-(mercuri)-p-toluenesulfonanilide.

Since absorption of lead may occur when it is combined with arsenic,[20,27] it appears that there may be some mechanism in cattle which retards, but does not prevent, the absorption of lead when it is associated with a more soluble metallic ion.

Heart.—Since many of the mercurial diuretics (even at therapeutic levels) may give rise to a variety of changes in cardiac rhythm and conduction,[9,19,41] a series of electrocardiograms were recorded from animal 1 throughout this experiment. Progressive degenerative changes from this animal's original pattern (fig. 6) were observed as the experiment progressed. The initial changes, seen on day 29, were a slurring of the ST segment and a depression of the T wave in leads II and III (fig. 7). By day 36 (fig. 8), there was a marked increase in potential, the T wave was depressed in all leads, and the duration of the QRS and T waves was considerably prolonged. The final electrocardiogram was made on day 37, one day before the animal's death, and the changes observed previously had become even further exaggerated (fig. 9).

The pattern was qualitatively similar to that produced by mercurial diuretics in man, dog, and cat[2,19] and supports Lehman's suggestion[19] that the cardiac effect is characteristic of mercurials in general, and not specifically of the mercurial diuretic group. The cardiac changes evoked by mercurials were originally reported[17] to result from vagal stimulation. However, Barker *et al.*[2] found that, when mercurials were administered to vagotomized dogs, the electrocardiogram apparently was similar to those obtained from dogs with intact vagi, and they concluded that mercury produces its effects by direct action on ventricular muscle. Histopathologic examination of the heart of animal 1 showed that the evidence of heart failure suggested by the electrocardiograms was the result of degenerative changes in the myocardial and Purkinje fibers.

Blood.—Erythrocytes.—The anemia reported to be produced by the fungicide methoxyethyl mercurisilicate[34] was not observed; both the number of erythrocytes and the hemoglobin levels (tables 4, 5) in the experimental animals remained within the normal ranges for the species[8] throughout the experiment. No structural changes of the cells attributable to the drug were observed in stain smears.

TABLE 3—Accumulation (mg.) of N-(ethylmercuri)-p-toluenesulfonanilide in Blood After Multiple Administrations

Animal No.	n*	Rate of administration	Predicted	Actual
1	38	0.0112 Gm./kg./day	41.330	44.730
2	14	0.056 Gm./kg./day	168.450	114.456
3	9	0.112 Gm./kg./day	572.100	189.550

* Number of repetitions.

Leukocytes.—It has been reported [35] that acute poisoning of cattle by methoxyethyl mercurisilicate produces neutrophilia with a "shift to the left." This blood change was not a feature in these animals. In animal 1, the total leukocyte count was increased between days 11 and 18, and a shift to the left occurred on days 16 and 18, concurrent with a nonacute, untreated infection; the shift was only transient. Lymphocytosis, which was also observed in methoxyethyl mercurisilicate poisoning, and agranulocytosis were seen terminally in the more acutely poisoned animals, (2 and 3, table 4).

Chlorides.—The blood chlorides were slightly and constantly elevated in animal 1 throughout the course of drug administration (table 5). In the more acutely affected animals, chloride levels were more variable. Although any marked increase in the blood chlorides of these animals was followed by a reduction, the average level was higher than that of animal 1, and of the species.[8] It is difficult, however, to ascribe any particular pathologic significance to the moderate chloride elevations observed in this experiment.

Blood Urea Nitrogen.—The BUN level increased terminally and rapidly in each case. Since water intake and the volume of urine excreted by animal 1 (table 7) did not vary markedly, even late in the course of administration, and since there was no evidence of edema, the elevated BUN level was attributed to acute renal failure rather than to inadequate fluid intake, fluid diversion, or excessive extrarenal output. At necropsy, there was glomerulonephritis and degeneration of the proximal tubules.

Serum Proteins.—There was a tendency, most marked in animal 3, toward the development of a moderate hypoalbuminemia. The γ globulin fraction was slightly decreased in animals 2 and 3, but the α and β globulin fractions were increased in all animals (table 6).

Subacute inflammation, particularly in dogs and swine, is associated with hypoalbuminemia and increased α and γ globulins.[5] In cattle, however, the subacute inflammatory processes produce hypoalbuminemia [30] with increased γ globulin but unchanged α and β fractions.[3] It therefore

TABLE 4—Hematologic Findings in Animals Given N-(ethylmercuri)-p-toluenesulfonanilide Experimentally

Day	Erythrocytes (million/cmm.)	Packed-cell volume (%)	Leukocytes per cmm.	Differential leukocyte counts (%)				
				Seg.	Band.	Lymph.	Mono.	Eosin.
ANIMAL 1								
2			7,000	30	12	57	0	1
4	6.6	....	7,300	44	2	54	0	0
9	4.0	....	9,000	23	16	61	0	0
11	5.3	35	13,400	0	18	80	0	2
16	7.1	40	20,000	33	30	36	0	1
18	6.1	35	17,000	25	28	46	0	0
23	5.4	40	5,300	22	9	67	0	2
30	8.5	40	7,800	31	11	52	0	6
32	7.2	40	6,000	14	13	62	0	11
36	6.2	36	5,500	16	10	72	2	0
37	7.1	40	9,600	25	19	56	0	0
38	6.7	45	14,600	20	36	44	0	0
ANIMAL 2								
1	7.4	40	11,900	35	7	50	1	7
3	7.9	39	11,800	22	10	68	0	0
6	7.9	45	12,850	10	10	71	1	8
11	7.5	47	9,000	3	5	90	0	2
14	8.8	53	11,400	1	7	91	0	1
ANIMAL 3								
2	6.1	38	7,800	25	19	60	1	3
4	6.5	41	8,000	5	6	85	0	4
7	9.8	68	4,800	4	14	80	0	2
9	10.1	56	5,050	....	....	....	..	..

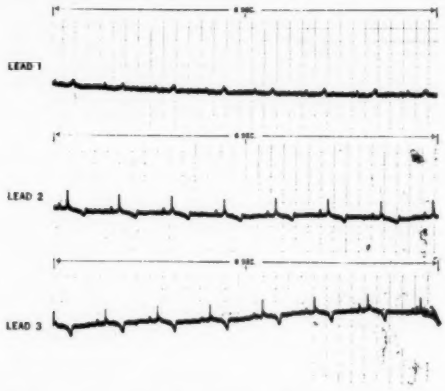

Fig. 6—A pretreatment record (animal 1) to establish the electrocardiograph pattern. Subject was standing.

appears that, in these animals, the reduced A/G ratio (table 6) was due to the action of the mercury rather than to an inflammatory response. A similar reduction in A/G ratio has been reported in chronic mercurial poisoning of man.[44]

Urine.—Volume.—The average daily urine secretion by a dairy cow is 14.2 kg., with a range of 8.8 to 22.6 kg.[10] and an average specific gravity of 1.030.[8] From these values, on a body weight basis, animal 1 (111 kg.) could have been expected to secrete an average of 3.6 liters per day with a range of 2.3 to 5.5 liters. The average daily secretion was found to be approximately 4.5 liters with a range of 2.7 to 7.0 liters (table 7).

In the absence of hemoconcentration in a nonedematous animal,[42] the increased urine secretion in this instance is interpreted as a response to irritation produced by repeated catheterization and a recurring urinary tract infection rather than to a diuretic action of EMTS.

Chlorides.—The urinary chlorides began to decrease on day 5, rose between days 25 and 31 (coincident with an acute urinary tract infection), but fell sharply terminally (table 7).

There were at least three mechanisms contributing to the disturbance of the chloride level in this animal. The developing glomerulonephritis (by its distortion of the glomerular vascular pattern) and the developing cardiac decompensation (by reduced hydrostatic pressure and volume blood flow) favored incomplete clearing of

TABLE 5—Biochemical Findings in Blood of Animals Given N-(ethylmercuri)-p-toluenesulfonanilide Experimentally

Day	Blood urea nitrogen (mg./100 ml. of blood)	Hemoglobin (Gm./100 ml. of blood)	Blood chlorides (mEq/L in serum)	Mercury (p.p.m./1 ml. of blood)
ANIMAL 1				
2	16	11.6	.....	N.D.
4	15	10.6	96	N.D.
9	14	7.8	116	0.25
11	12	9.2	120	0.6
16	12	10.1	121	0.6
18	11	8.9	126	....
23	10	9.8	120	1.0
26	20	.....	105	....
30	18	10.8	125	2.0
32	18	10.2	115	3.5
36	14	8.1	114	4.5
37	33	11.6	111	5.7
38	74	10.8	117	7.1
ANIMAL 2				
1	9	13.1	115	....
3	14	14.1	158	....
6	16	15.3	107	....
7	....	.....	.....	4.6
11	29	14.3	111	12.0
14	75	16.1	104	22.8
ANIMAL 3				
2	13	14.6	111	....
4	....	12.4	147	6.93
7	42	16.4	100	19.25
9	91	16.2	137	22.3

N.D. = not detectable.

TABLE 6—Results of Serum Protein Analyses in Animals Given N-(ethylmercuri)-p-toluenesulfonanilide Experimentally

Day	Serum protein (%)				
	Albumin	Globulins			A/G ratio
		α	β	γ	
ANIMAL 1					
36	46.4	16.1	17.8	19.7	0.87
37	43.9	21.6	15.1	19.4	0.78
38	34.5	21.1	24.6	19.8	0.53
ANIMAL 2					
1	50.7	19.4	16.6	13.3	1.03
7	44.1	21.9	18.9	15.1	0.79
14	42.3	27.2	19.4	11.1	0.73
ANIMAL 3					
1	61.5	13.5	11.1	13.9	1.60
7	43.5	25.9	20.7	9.9	0.77
8	36.4	35.2	16.4	12.0	0.57

efferent glomerular blood. Generally, these two factors combined to more than offset the opposing influence of an increased filtration rate which occurred as a response to lower urinary tract irritation. When acute infection occurred between days 25 and 31, the rate of urine secretion doubled. Although the total chloride excretion increased as a result of the increased filtration, the per cent of excretion remained well below the normal range (table 7).

Specific Gravity.—The specific gravity was reduced from the third day onward, becoming fixed between 1.005 and 1.015 g (table 7). These lowered values were a reflection of the increased fluid excretion and the loss of concentrating power by the kidneys.

Nervous System.—The EMTS produced degenerative changes in both the brain and cord of each of the experimental animals. In the cerebrum, diffuse neuronal degeneration, gliosis, and focal areas of malacia were observed. Many of the Purkinje cells of the cerebellum had degenerated, cortical hemorrhages were present, and there was gliosis of the molecular and granular

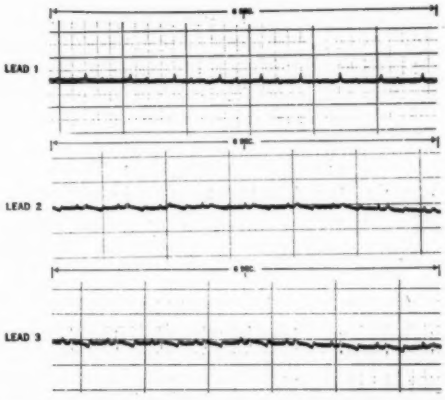

Fig. 7—First signs of change in electrocardiograph pattern, seen on day 29. The subject was standing. Slurring of the ST segment and depression of the T wave are seen in leads II and III.

layers. The myelin sheaths in the cervical, thoracic, and lumbar regions of the cord were edematous and hemorrhages were present in the dorsal horn of the cervical area.

In view of the apparent lipotropicity of the compound, based on its high concentration in nerve tissue (table 1), the neuro-

TABLE 7—Urine Findings in Animal 1

Day	Volume (liters)	Total chlorides (Gm.)	Chlorides (%)	Specific gravity	Albumin	Sugar	Mercury	
							p.p.m./ml.	mg. per 24 hr.
Pretreatment values		27.39 ±4.7	0.868 ±0.12	1.020	0	0	N.D.	
2	2.7	30.34	0.95	1.020	0	0	N.D.	N.D.
3	5.0	29.24	0.58	1.015	0	0	N.D.	N.D.
4	....	.....	0.79	1.015	0	0	N.D.	N.D.
5	4.0	18.05	0.45	.....	0	0	N.D.	N.D.
9	5.5	16.10	0.29	1.0075	0	0	.....	.....
10	4.1	9.94	0.24	1.011	0	0	0.05	0.19
11	3.6	16.0	0.45	1.012	0	0	.....	.....
12	3.1	16.85	0.54	.....	+	0	0.2	0.62
16	3.4	15.01	0.45	1.008	+	0	.....	.....
17	6.4	20.73	0.33	1.010	+	0	.....	.....
25	6.0	33.04	0.43	1.015	+	3+	0.35	2.45
26	5.3	21.87	0.41	1.021	+	3+	.....	.....
31	7.0	27.02	0.34	1.005	+	3+	0.6	4.8
33	4.2	6.31	0.15	1.010	+	+	0.7	2.9
38	....	.....	0.112	1.012	3+	—	2.75	.....

N.D. = not detectable.

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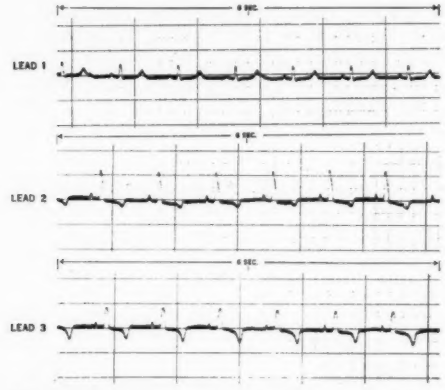

Fig. 8—The gross changes in pattern are evident (day 36). The subject was standing. The duration of QRS and T waves are markedly prolonged, the ST segment is slurred, and T waves are depressed in all leads. The potential increased.

psychosis which resulted developed rather slowly. Hughes [16] considered that the slow onset of nervous signs indicated that mercurials do not inhibit nervous function directly but act by blocking the metabolism of the neurons by producing irreversible degenerative changes.

The predilection of mercurials for nervous tissue appears to vary with the compound and the species. The methyl mercurials [22] produced degenerative changes in the brain and cord of the monkey but caused a precise localized degeneration in the brain of man. Ethylmercuric chloride*

* Ceresan, produced by E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.

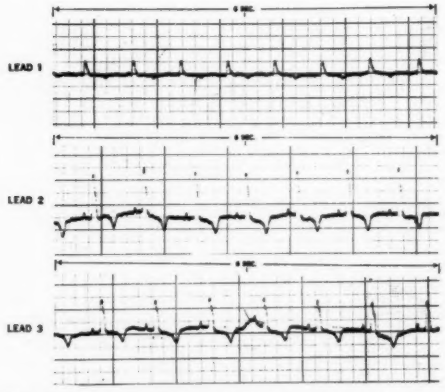

Fig. 9—Changes observed on day 37 are further exaggerated. The subject was recumbent.

did not affect the central nervous systems of cattle with either subacute or chronic poisonings,[35] but, as reported here, EMTS caused severe, diffuse lesions in this species at both subacute and chronic levels.

In the rat, mercuric chloride produced specific cerebellar lesions,[11] whereas, in the same species, Swensson [37] found that five organomercurial compounds caused diffuse injuries in the central nervous system without any essential differences in toxicity between the substances tested. Mueller [24] was unable to detect mersalyl in the brain of a dog two hours after intravenous administration. Shoemaker,[33] reviewing the pharmacology of mercury and its compounds, concluded that the mercurial diuretics are not lipotropic and thus have no toxicity for the central nervous system. Including EMTS, the toxicity for animals of eight organomercurial seed disinfectants has been reported.[35,37,38] None of these contain the propyl methoxy radical (—CH_2COCH_3 CH_2—) common to the mercurial diuretics. Seven of the eight preparations caused central nervous disturbances [37,38] but the eighth, ethylmercuric chloride, which contains an ethyl methoxy (—$CHOCH_3$ CH_2—) had no toxic effect on the nervous tissue in either subacute or chronic poisoning. These results suggest that the neurotoxicity of an organomercurial compound may be related to the absence of an alkyl methoxy on the molecule.

SUMMARY

N-(ethylmercuri)-*p*-toluenesulfonanilide (EMTS) produced signs of central nervous system or gastrointestinal disturbance, or both, in cattle. The nature of the signs depended on the rate of administration. In absolute amounts, the absorption rate of EMTS increased at an increasing rate. Logarithmically, there was a fixed rate for a given dose which increased linearly with increased dosage. Following a single administration, the compound reached a peak concentration in the blood in approximately 30 hours. It was transported by the plasma but chiefly by the red blood cells; *in vitro*, 1M of plasma albumin bound with 2M of EMTS. It was unequally distributed throughout the body and reached its greatest concentration in the kidneys. It caused progressive degenerative changes in the heart, moderate hypoalbuminemia, and reduced blood A/G ratio. It produced diffuse lesions in the cord, cerebellum, and

cerebrum and caused glomerulonephritis. The possible biological significance of the alkyl methoxy radical in the molecules of certain organomercurial compounds is considered.

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SUMMARIO IN INTERLINGUA

Studios in le Pharmacologia de N-(ethylmercuri)-p-toluensulfonanilido

In bestial, N-(ethylmercuri)-*p*-toluensulfonanilido (EMTS) produceva signos de disturbationes del systema nervose central o del apparato gastrointestinal. Le natura del signos dependeva del mesura del administration. Exprimite in cifras absolute, le rata del absorption de de EMTS cresceva a un rata crescente. Logarithmicamente, il existeva un rata fixe de absorption pro un dose particular. Con augmentos del dose, ille rata cresceva linearmente.

Post un sol administration, le composito attingeva su maximo de concentration in le sanguine intra approximativemente 30 horas. Illo esseva transportate per le plasma e, principalmente, per le erythrocytos. In vitro, 1 M de albumina del plasma se ligava con 2 M de EMTS. Le distribution de EMTS in le corpore non esseva uniforme. Le concentration maximal esseva constatate in le renes. Le composito causava progressive alterationes degeneratori in le corde; illo effectuava grados moderate de hypoalbuminemia; e illo reduceva le proportion de albumina a globulina in le sanguine. Illo produceva diffuse lesiones in le medulla dorsal, le cerebello, e le cerebro. Illo causava glomerulonephritis. Le possibile signification biologic del radical alcoyl-methoxylic in le moleculas de certe compositos organomercurial es discutite.

Scientific Notes

A Preliminary Report on the Variation in Numbers of Nematode Eggs Demonstrated in Individual Fecal Pellets of Sheep

EVERETT D. BESCH, D.V.M., M.P.H.; ROBERT D. MORRISON, B.S., M.S., Ph.D.; DAVID L. WEEKS, B.S., M.S., Ph.D.

DEVELOPMENT of methods to qualitatively demonstrate the presence of helminth eggs in feces has enabled parasitologists to obtain estimates of the composition of gastrointestinal parasite populations of domestic animals. Furthermore, demonstrations of helminth eggs in feces on a quantitative basis have been used extensively to study fluctuations in helminth populations. Regardless of the egg-counting method used to determine the number of nematode eggs in the feces of individual herbivores, the results obtained may vary considerably. This variation can be attributed to numerous factors, other than technique, which may be referable to the normal physiology of either the host or the parasite, or both.[4,5,10] Because of these variables, it is difficult at times to interpret the value and significance of quantitative egg counts in the study of host-parasite relations. This is a report on a preliminary study to determine the magnitude of variation that may occur in nematode egg counts when using individual fecal pellets of sheep as the unit of feces to be measured.

Several reports describing the variation in worm-egg content of sheep feces have been published; however, none of these accounts were concerned with the variation that may occur in the number of eggs in individual sheep pellets as determined by the small-drop-dilution, egg-counting method. For counting worm eggs in sheep feces, 10-Gm. units of feces have been utilized.[9] Use of one unweighed fecel pellet from each sample has been advocated for determining the number of eggs per gram in sheep feces.[6] Because of differences in the consistency of their feces, this method was not found to be satisfactory when used for egg counts of feces obtained from animals which were on pasture.[8] Variation in the egg content of different samples of sheep feces collected within a 24-hour period and the variation from day to day have been studied.[7,8] These data indicated that egg counts from different fecal samples taken within a 24-hour period had considerable variation; therefore, it was considered difficult to rely on the count of a single sample taken within a day from any single animal. To reduce the effect of this variation, use of the egg per day count in reporting data was recommended, because it gives a direct measure of the egg output of the worm population over a 24-hour period. The egg per gram count, which is the unit of measurement usually used in reporting, is an index of this total distorted by variations in fecal sample weights and is subject to other factors which are independent of the feces. Presentation of data as worm eggs per gram has been almost universally adopted and should be used in presenting data since it is used by most workers. Any other method of reporting such results could possibly lead to attributing greater significance to the transformed data.[1]

MATERIALS AND METHODS

Fecal samples were obtained daily, by use of a canvas collection bag, from a mature ewe. At the end of each collection period, the sample was weighed, the moisture content was determined, and individual fecal pellets were selected at random for nematode egg count determinations. The egg counting method used was a modification of the techniques described by Stoll.[9] This method, which may be adapted to small-unit, fecal-sample weights, has a feces-decinormal sodium hydroxide solution ratio of 1 in 30.

RESULTS

Statistical analysis of the data obtained from egg counts of 88 individual fecal pellets indicated that the range of egg count variation attributable to technique was 5.8 to 19.6 per cent, while the range of variation in the total number of eggs in individual pellets (assuming no variation in technique) was 2.4 to 17.8 per cent, with three estimates being negative.

Weights of individual pellets and their moisture content were determined from samples that were collected at intervals while the ewe was maintained on two separate rations. When prairie hay was fed, the weight range of 120 pellets, selected at random, was 0.31 to 1.58 Gm., with a mean of 0.88 Gm. The moisture content of 27 of these fecal samples was 44.0 to 58.8 per cent, with a mean of 53.3 per cent. When a ration of prairie hay and

From the Department of Veterinary Parasitology (Besch), College of Veterinary Medicine, and the Department of Mathematics, College of Arts and Sciences (Morrison and Weeks), Oklahoma State University, Stillwater. Approved for publication as journal manuscript No. 563 by the director of the Oklahoma Agricultural Experiment Station.

Portions of this study were conducted as part of Southern Regional Project S-21, supported in part by regional funds of the USDA.

grain supplement was fed, individual pellets weighed 0.62 to 5.23 Gm., with a mean of 1.64 Gm. The moisture content of 48 of these samples was 56.8 to 83.5 per cent, with a mean of 67.2 per cent.

DISCUSSION

The significance of quantitative egg counts depends, in part, on both the accuracy of methodology and observation. Further studies are necessary to determine the source of the variation due to technique and the amount of variation in the actual total number of nematode eggs present in individual ovine fecal pellets.

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SUMMARIO IN INTERLINGUA

Reporto Preliminari Relative al Variation del Numero de Ovos de Nematodo Demonstrabile in Granos Individual de Fece Ovin

Es discutite le problema del determination quantitative de ovos de nematodo in fece ovin. Resultatos preliminari es presentate super le base de numerationes in granos de fece individual. Es notate variationes de grado notabile. Le conclusion es que studios additional es necessari pro determinar le origine del variabilitate in tanto que illo es causate per factores technic, per factores contribuite per le animal hospite, e per factores in le comportamento del nematodos.

IVth International Congress On Animal Reproduction

The IVth International Congress on Animal Reproduction will be held at The Hague, Netherlands, June 5-9, 1961.

The provisional subjects for the morning sessions are: artificial insemination of pigs; infection and infertility; biochemistry of the ovum and spermatozoa; the influence of prenatal environment on the fetus in poultry and mammals; experience with mass examination of bulls for fertility; and reproduction as a function of breed and environment. The sectional meetings on physiology, pathology, and artificial insemination will be held in the afternoons. Films, demonstrations, exhibits, and excursions will be included in the program.

Those interested in contributing reports should notify the secretariat before Oct. 31, 1960; deadline for submissions is Jan. 15, 1961. For further information, write to the Secretariat, IVth International Congress on Animal Reproduction, 14 Burgemeester de Monchyplein, The Hague, Netherlands.

Instrument Symposium and Research Equipment Exhibit

The 10th Annual Instrument Symposium and professional societies, will be held Oct. 3-6 and Oct. 4-7, 1960, respectively, at the National Institutes of Health, Bethesda, Md.

The scientific sessions of the instrument symposium will be held twice daily throughout the week. The program will include reports and discussions on the biological applications of fluorescence, infrared spectrophotometry, activation

analysis, ultracentrifugation, electrophoresis, microscopy, and membrane electrodes.

Research appliances and related products will be displayed by 120 manufacturers at the equipment exhibit, open daily. In addition, special instrument clinics have been scheduled for morning sessions. Representatives of six manufacturing firms will demonstrate the research applicability of equipment such as an electronic counter, physiological gas analyzer, fluorescent microscope, robot chemist, recording balance, and nitrogen analyzer.

For additional information, contact James B. Davis, National Institutes of Health, Public Health Service, Bethesda 14, Md.

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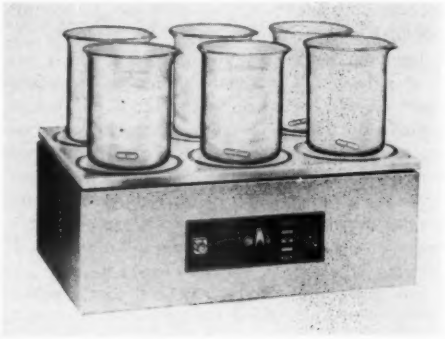

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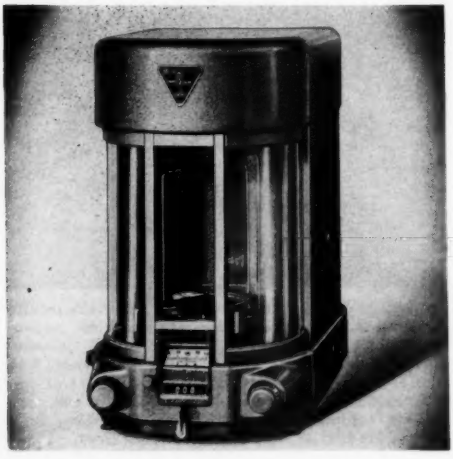
The FW 55 Universal high-speed balance, manufactured by Mikrowa, is distributed in the U.S. by the C. H. Stoelting Co. The direct-reading electro-optical scale of 10,200 mg. (10.2 Gm.) is combined with an automatic weight-placing system which is operated by two dials. The balance has a capacity of 200 Gm., with an accuracy of 1 mg. and a reproducibility of 0.5 mg.



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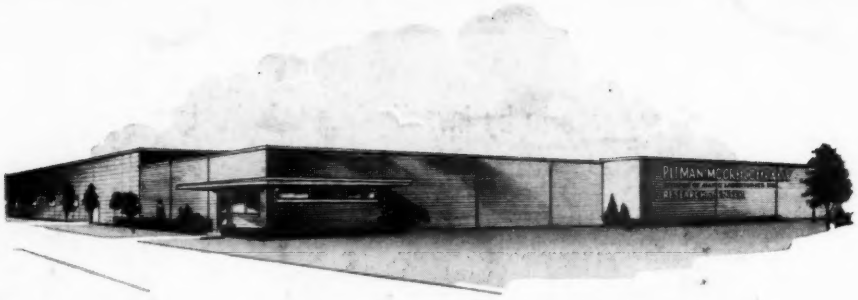

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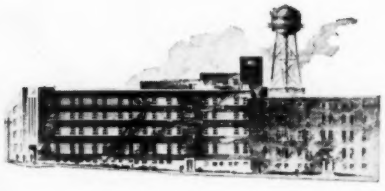

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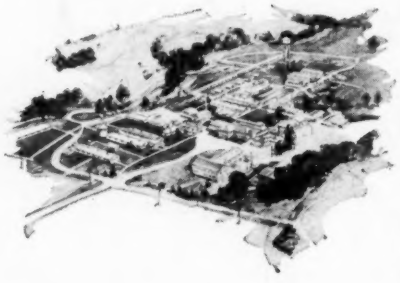

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AVMA RESEARCH FELLOWSHIPS AVAILABLE

The Council on Research of the AVMA announces the availability of a number of fellowships for postgraduate training for the academic year, 1961 - 1962.

The recipient of a fellowship must be a veterinarian and a citizen of the United States or Canada. Veterinary students who expect to graduate at the end of the current school year and who wish to follow a career in research may apply for a fellowship.

It is advisable that completed application forms be filed by Jan. 1, 1961, to allow time for correcting omissions and for some reasonable delay in arrival of letters of information from third parties. The latest date for filing the completed application is Feb. 15, 1961. Approximately one month is required for processing completed applications after receipt by the Council. Qualified persons should secure and submit applications as early as possible to insure their file being complete for presentation to the Committee on Fellowships.

The Committee on Fellowships of the Council on Research will meet early in March to consider applications, and the awards will be announced soon afterward. The stipend will be determined in each case by the needs of the individual, the location of the school in which he proposes to work, and other factors. In general, the stipends range from $100 per month upward.

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1. Osborne, J. C. *in* New Horizons in Chemotherapy. Proceedings of First Regional Conference on the Nitrofurans in Veterinary Medicine. In press.
2. Bull, W. S.: N. Amer. Vet. 38: 3 (Jan.) 1957.

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